CALCIUM METABOLISM IN A CASE OF GARGOYLISM, STUDIED WITH THE AID OF RADIOCALCIUM [1]

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A number of studies have been reported on the fate of radiocalcium (Ca-45) in humans (1–12), but none have included chemical analyses of the concentration of Ca-45 in the skeleton and in such tissues as liver, brain, or intestinal tract. We are now reporting the results of a metabolic study with Ca-45 performed on a 10 year old patient with terminal gargoylism. The analyses of periodic samples of serum, urine, cerebrospinal fluid, and feces obtained during the patient's illness and of various tissues removed at necropsy 16 days after the administration of Ca-45 have made it possible to formulate a relatively complete picture of the calcium metabolism of this individual, although the lack of comparable information makes it difficult to evaluate whether and how gargoylism modified the calcium metabolism of our patient.

Gargoylism is now well recognized clinically (13), but its pathogenesis is largely unknown. Evidence is accumulating (14–19) to indicate that gargoylism is a storage disease, characterized by the accumulation of abnormal types (20) and quantities of mucopolysaccharides in a large variety of tissues, notably liver and spleen.

Recently, it was proposed that polysaccharides, such as chondroitin sulfate, are involved in the calcification process in bone and cartilage [see Amprino (21) for a review of the evidence]. Although gargoylism appears to be characterized by a defect in structural polysaccharides, it is possible that calcification is also defective in this condition. Dawson (17) has reviewed the histological changes seen in the bones of patients with gargoylism and has concluded that the changes are dystrophic and are probably of direct metabolic origin. However, no metabolic studies have been reported. It seemed of interest, therefore, to study in detail the calcium metabolism of a patient with terminal gargoylism.

MATERIAL AND METHODS

Description of patient. The patient was a male child, 9 years 10 months old, 137 cm. high, who weighed 16.4 Kg. He had been institutionalized since the diagnosis of the disease almost five years earlier. When the study was initiated, the disease had progressed to a point where the boy's life expectancy appeared short. The patient was almost completely unaware of his surroundings, had lost his ability to communicate except by a bird-like whine, and showed no response to sounds. He appeared incapable of any muscular control, was incontinent of urine and feces, and had to be handled like a 3 months old baby. His arms and legs were flexed, with ankylosis of the joints. The hands were clenched, the fingers showed clubbing, and the skin was taut and shiny. There was general hirsutism.

Design. Although the patient was on the danger list, his condition had remained unchanged for several weeks preceding the study. It was planned to follow the course of the injected Ca-45 intensively during the first five days, then at weekly, and later at monthly intervals. The death of the patient on Day 16 prevented an extended follow-up.

Dietary regimen. Before the initiation of the study and throughout its duration, the patient was spoon-fed with commercial baby foods, baby cereals, and milk. No attempt was made to regulate his intake. During the course of the study, *i.e.*, on days when specimen collection was carried out, food equivalent to that fed to the patient was collected by having the nursing personnel place in a collection jar quantities of food identical to those fed the patient. His calcium intake was analyzed as 3.8 Gm. on Day 1, 5.2 Gm. on Day 2, 4.3 Gm. on Day 3,

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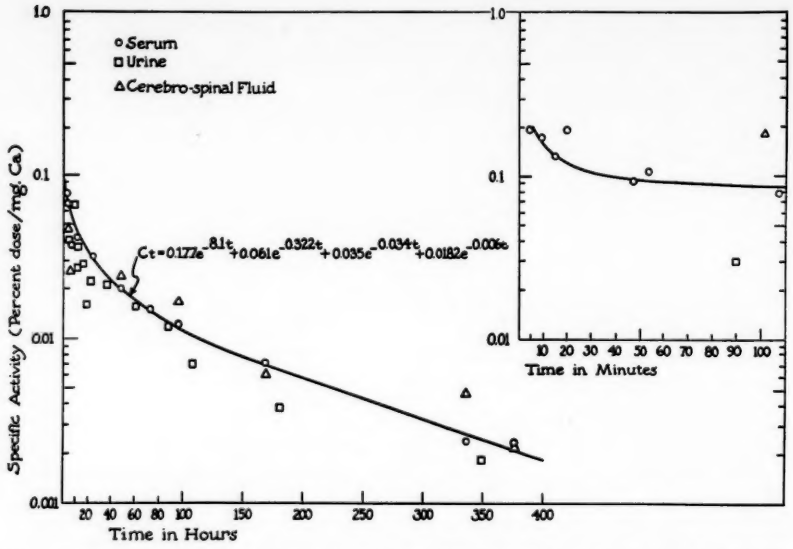

FIG. 1. SPECIFIC ACTIVITY OF SERUM, URINE AND CEREBROSPINAL FLUID OF A PATIENT WITH GARGOYLISM FOLLOWING INTRAVENOUS INJECTION OF 80 μC. OF $CA\text{–}45Cl_2$

The equation predicts the course of the specific activity of the serum with time. C_t equals per cent dose per mg. Ca, t equals hours. Average calcium content of serum: 0.085 mg. per ml.; range, 0.081 to 0.090. Average calcium content of cerebrospinal fluid: 0.049 mg. per ml.; range, 0.044 to 0.059.

1.3 Gm. on Day 4, 1.0 Gm. on Day 5, 0.5 Gm. on Day 8, and 3.2 Gm. on Day 15.[4]

Calcium-45.[5] The Ca-45 was diluted in a solution containing 0.8 per cent of NaCl and 0.1 per cent of $CaCl_2$. A total of 3 ml. of solution was injected intravenously; it contained 80 μc. of Ca-45, 1 mg. of Ca^{++}, 10 mg. of Na^+, and 16 mg. of chloride, and had been sterilized by autoclaving.

Specimen collections. Blood was obtained by venipuncture at intervals indicated in Figure 1. Samples of *cerebrospinal fluid* were obtained by spinal puncture (Figure 1). *Urine* samples were obtained from the catheterized patient at three hour intervals during the first day; thereafter (Days 2 to 5, 8 and 15), 24 hour specimens were collected. Following collection, the urine was acidified by the addition of glacial acetic acid in the proportion of 2 ml. per 100 ml. urine. *Feces* specimens were collected on diapers. On the first day, these were pooled to constitute eight hour specimens; on subsequent days, 24 hour specimens were analyzed.

Autopsy. Representative samples of mineralized and soft tissues were removed at autopsy and analyzed (see Tables I to III). In the case of certain hard tissues (Table II), the analysis of the whole section was supplemented by analysis of scrapings, obtained with a dental burr at anatomically defined regions (22, 23).

Analytical procedures

Calcium analysis. Blood calcium content was determined by direct precipitation of the calcium from the serum as the oxalate, which was titrated with perchloratoceric acid (24). Urine calcium was determined by precipitation of the calcium either directly from the urine or from an ash solution of the urine. Calcium contents of stool specimens were determined on fecal ash solutions. The coefficient of variation (standard deviation times 100 divided by the mean) of the calcium determinations was usually below 5 per cent.

Ca-45 analysis. Ca-45 was determined by measuring the radioactivity of calcium oxalate samples which contained 4.0 mg. of Ca. The solid samples were counted under a thin end-window (< 1.5 mg. per cm.^{2}) Geiger-Müller counter, connected to an automatic sample-changing device. Suitable standards were employed in all counting runs and all results are reported as corrected for decay from the day of injection. Counting errors varied.

[4] An estimate of the calcium content of the food eaten, based on the nurses' notes, would indicate that these surprisingly high values for Ca intake are reasonable, as the estimated cumulative Ca intake is only about 17 per cent lower than the analytical value.

[5] The Ca-45 was obtained on allocation from the Oak Ridge Installation of the Atomic Energy Commission. Authorization for the use of 80 μc. of Ca-45 in a moribund patient with gargoylism was granted through the Sub-Committee on Human Applications.

but their coefficients of variation were generally less than 10 per cent. The term "dose" refers to the quantity of Ca-45 injected.

The analytical techniques employed have been described in detail by Bronner, Harris, Maletskos, and Benda (3).

RESULTS

Serum

When the serum data are plotted on semilogarithmic coordinates (Figure 1), they fall on a complex curve which is approximated by the following equation:

$$C_t = 0.177e^{-8.1t} + 0.061e^{-0.322t} + 0.035e^{-0.034t} + 0.018e^{-0.006t}, \quad 1)$$

where

C_t = Per cent of dose per mg. of Ca in serum

and

t = Hours.

TABLE I

Specific activity of selected soft tissues obtained at autopsy of a patient with gargoylism who had received by intravenous injection 80 μc. of $Ca\text{-}45Cl_2$ 16 days previously

Tissue	Ca-45 content (% dose/Gm. Ca)
Serum	2.26
Brain	3.06*
	3.17
Spleen	2.79
Kidney	1.55
Liver	2.52
	3.08
Diaphragm	1.67
Intestinal tract	
Duodenum	1.57
	2.10
Jejunum	2.04–2.82†
Ileum	2.42
	3.26
Ascending colon	1.51–1.76
Transverse colon	1.25
Descending colon	2.10
	1.44
Sigmoid flexure and rectum	1.44
	1.83
Bile	2.92 (Ca content: 0.692 mg./ml.)
Washings	
Small intestine	1.54
Colon	0.75
Sigmoid flexure and rectum	1.01

* Two entries indicate determinations on two samples of tissue.

† A dash indicates the range of determinations on more than two samples of tissue.

TABLE II

Ca-45 contents of bone scrapings obtained at autopsy of a patient with gargoylism who had received by intravenous injection 80 μc. of $Ca\text{-}45Cl_2$ 16 days previously

Tissue, sample no., and description*		Ca content (mg. Ca/Gm.)	Ca-45 content (% dose/Gm. Ca)
Calvarium			
A1		251	0.083
A2		228	0.071
A3		221	0.143
A4		211	0.195
A5		194	0.217
A6		239	0.166
Premolar tooth, unerupted			
E1	Enamel	372	0.0205
E2		349	0.0180
E3		338	0.0155
DEJ	Dentino-enamel junction	428	0.0137
D1	Dentin	312	0.000
D2		299	0.0780
D3		366	0.040
D4	Internal dentin	386	0.1047
Ulna			
C1	Spongy bone, distal head	294	0.119
C2	Endochondral bone, distal head	231	0.089
C3	Subepiphyseal region	287	0.178
C4	Trabecular spicules	228	0.133
C5	Spicules	265	0.106
D2	Subsurface cross segment	203	0.217
D3	Central cross segment	389	0.220
Ulna, shaft			
B1		320	0.0572
B2		213	0.0809
B3		294	0.0497
B4		322	0.0448
Ulna, proximal end			
E1	Subepiphyseal	222	0.212
E2	Trabecular	153	0.170
E3	Subperiosteal funnel region	215	0.186
Metacarpal bone			
Central		223	0.184
Marrow area		240	0.151

* See Figure 2 for location of sampling sites.

This equation is not valid for the first five minutes of the study and therefore its first term has little significance. Its general course resembles that observed in other children (5, 7), but differs from these significantly (Table IV), inasmuch as the exponents of the second and third terms of equation *1*) are only about one-tenth of the corresponding terms of the normal boys, and are also smaller than those of the adult. The exponent of the fourth term is as fast as that of the fourth term in the equation of the adult, but much slower than

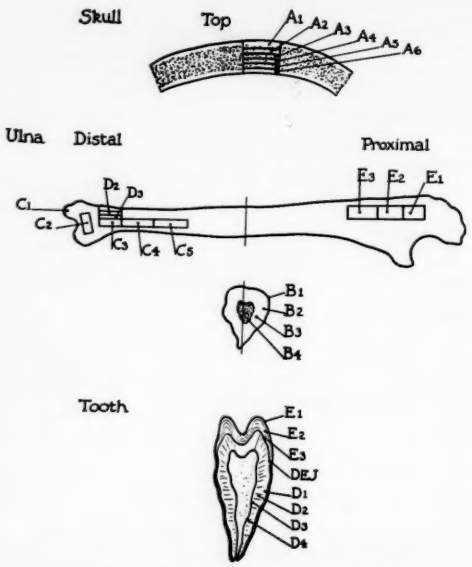

FIG. 2. DIAGRAMMATIC LOCALIZATION OF SAMPLING SITES FOR ANALYSES SUMMARIZED IN TABLE II

Samples were obtained with the aid of a dental burr.

TABLE III

Specific activity of selected hard tissues obtained at autopsy of a patient with gargoylism who had received by intravenous injection 80 μc. of Ca-45Cl₂ 16 days previously

Tissue	Ca content (*mg. Ca/Gm.*)	Ca-45 content (*% dose/Gm. Ca*)
Spine, thoracic section	106	0.155
	102	0.138
Sixth left rib	152	0.197
Sixth right rib	194	0.195
Seventh left rib	158	0.198
Seventh right rib	111	0.217
Sternal cartilage, left	1.08	1.45
Sternal cartilage, right	1.90	1.76
Sternum	47.7	0.460
Skull, calvarium	82.4	0.271
Great wings, sphenoid bone	172	0.178
Left ulna		
Posterior end	123	0.202
Posterior shaft	119	0.088
Anterior shaft	125	0.102
Anterior end	189	0.139
Metacarpal bone	75.6	0.329
Alveolar bone	314	0.215
Teeth		
Upper left deciduous canine	354	0.054
Lower left deciduous canine	381	0.025
Upper right deciduous molar	328	0.033
Lower right deciduous molar	331	0.012

TABLE IV

Comparison of exponents in serum disappearance equations *

Boys†	Young adult†	Gargoyle patient
0.281	0.713	0.135
0.075	0.0112	0.0054
0.004	0.0004	0.00057
0.0005	0.00009	0.00010

* Dimension: minute^{-1}.
† Taken from Bronner and Harris (7).

the corresponding exponent in the equation of the normal boys.

A comparison of the last term of equation *1*), with the coefficients, rate constants, and compartment sizes calculated by Krane, Brownell, Stanbury, and Corrigan (8), shows that the last term, with an intercept of 18.2 per cent dose per Gm. Ca and a slope of 0.138 per day, agrees well with the third term (Days 4 to 9) of equations reported by these authors for euthyroid adults. Similarly, the "calcium pool," 16.7 Gm., (per cent activity retained at Day 9 divided by the serum specific activity on Day 9) of our subject was comparable to that shown by Krane and collaborators (8) for euthyroid adults. However, on a body weight basis, the "pool" was much greater in our subject than in normal adults, as would be expected from the higher calcification rate seen in children.

Comparison of terms 2 and 3 of equation *1*), with the comparable coefficients and rate constants of Krane and co-workers (8), shows (see also Table IV) that the processes which have given rise to these terms in our patient differ markedly from those observed in normal adults or normal children.

Urine and feces data

As would be expected, the specific activity data of serum and urine (Figure 1) can be considered to fall essentially on one curve, although the corresponding values for the urine tend, perhaps, to be lower than those for the serum. The very low output of calcium in the urine (Figure 3) may bear on this point.

The endogenous fraction of fecal calcium during the first five days is estimated from the mean serum specific activity during that period, on the assumption that the specific activity of the se-

creted intestinal juice always equals that of the serum (5). As the mean specific activity of the serum between 0 and 120 hours was 0.0233 per cent dose per mg. Ca, and as our patient had excreted 9.70 per cent of the dose in his stools during that period, his endogenous calcium output was 416 mg. or 11.0 per cent of the total calcium output in the feces. This value is similar to that observed by us in other instances (7).

In contrast with observations on normal, ambulatory patients (1, 5) or on terminal cancer patients (2, 4), the ratio of total urinary to fecal Ca-45 output was very much below unity (Figure 3). Geissberger (1) has reported ratios of this magnitude for patients with cardiac insufficiency and steatorrhea, as have Laszlo and Spencer [quoted by Comar and Wasserman (9)] for two patients with cancer of the prostate.

Cerebrospinal fluid

The specific activity of the spinal fluid approached that of the serum between 5 and 48 hours after injection (Figure 1). Thereafter, its specific activity either was equal to or higher than that of the serum. Postmortem analysis of brain tissue indicated a specific activity of 0.031 per cent dose per mg. Ca, as opposed to the serum specific activity of 0.023 at the time of autopsy. This difference gives support to the possibility that the specific activity of the cerebrospinal fluid may actually have exceeded that of the serum.

Bile

Possibly the serum was diluted with calcium released by autolysis during the period which elapsed between death and autopsy (about seven hours); otherwise, the specific activities of bile fluid and serum ought to have been more nearly alike than was found (Table I). The observed specific activity of the bile fluid was of the same order of magnitude (0.002 per cent per ml.) as reported for adult rats (25).

Singer, Maqsood, Medlen, and Comar (26) have very recently reported on the endogenous and biliary excretion of Ca-45 in dogs. Their data indicate that, within 25 minutes of injection, 21 per cent of the activity found in the contents of the gastrointestinal tract (excluding the stomach) is found in the gall bladder. Our data show that the total activity found in the bile approximated 27 per cent of the activity washed out of the gastrointestinal tract, excluding the stomach (Table I).

Soft tissues

The specific activity of the small intestine increased in a posterior direction. Contrary to expectations, the relative specific activity of the intestine was not highest near the point where the common bile duct enters the duodenum (7 to 8 cm. posteriorly of the pylorus). On the other hand, the low relative specific activity of the washings of the intestinal tract (Table I) suggests that even at a late stage in the patient's illness—when his food intake was rather small—labeled body calcium was still being diluted to a fair degree by unlabeled exogenous calcium.

Of some interest is the observation that several soft tissues (brain, spleen, liver, sections of the ileum and jejunum) had a higher specific activity than serum (Table I). Bronner (27) has recently shown that in very young rats the specific activity of many tissues is higher than that of serum. Inasmuch as the serum supplies tracer to a multi-

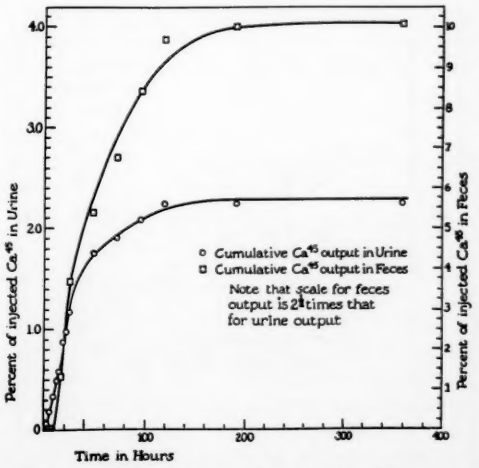
Percent of injected Ca45 in Urine
Percent of injected Ca45 in Feces
Time in Hours
Cumulative Ca45 output in Urine
Cumulative Ca45 output in Feces
Note that scale for feces output is 2½ times that for urine output

FIG. 3. CUMULATIVE OUTPUT OF CA-45 IN URINE AND FECES OF A PATIENT WITH GARGOYLISM FOLLOWING INTRAVENOUS ADMINISTRATION OF 80 μC. OF $CA\text{-}45Cl_2$

Average calcium output per day, first five days: urine, 23 mg.; range, 10 to 40; feces, 755 mg.; range, 470 to 1,065. On Days 8 and 15, the output of calcium dropped to 3.4 and 4.1 mg. in the urine and to 226 and 104 mg. in the feces, respectively.

compartment system, it is not surprising that its specific activity drops below that of some compartments.

Mineralized tissues

The data in Tables II and III are of interest because they indicate the fate of a single dose of Ca-45 in relation to the architecture of the bones. It is apparent, as has been reported by others (28–36), that the most highly calcified areas contain the smallest quantity of isotope. Thus, the extremities of the ulna showed more labeling than the shaft, undoubtedly because growth was still proceeding at the epiphyses, whereas the center of the shaft had stopped growing and was probably subject to remodelling only. The sternal cartilage of the ribs had a specific activity which was near that of the serum and which was approximately ten times as high as that of the bones [*cf.*, Kulp, Eckelmann, and Schulert (37) for similar observations on strontium deposition].

DISCUSSION

The much slower rate of disappearance of Ca-45 from the serum of our patient, as compared with that of normal boys (5, 7), suggests a disturbed calcium metabolism due perhaps to interference with calcium deposition or accretion. It is not surprising, therefore, that evaluation of A ("accretion value") in accordance with the method of Bauer, Carlsson, and Lindquist (38) [see Bronner and Harris (7) for a description and discussion of this approach] reveals an average value of 0.54 Gm. per day. This is about one-fourth of the value calculated for a group of boys, and less than the value calculated for two young men (7). The average E value, which represents the exchangeable fraction of body calcium (7, 38) of our patient, was 2.8 Gm., or 0.93 per cent of an estimated body calcium content of 300 Gm.[6] This value is comparable to those we calculated for other subjects (7).

Conceivably, the inability to deposit calcium, as indicated by the accretion value, is related to the accumulation of abnormal polysaccharide materials similar to those reportedly found in liver and spleen (16, 18–20). Chondroitin-like materials have recently been reported to occur in bone (40).

Because our patient's urinary output of Ca-45 was depressed, though his output of endogenous calcium in the feces was within the normal range, his absorption of calcium may have been altered, but his death precluded further studies.

If the data of Table II are plotted on semilogarithmic coordinates (Figure 4) they fall along a straight line which is expressed (method of least squares) by the equation

$$C = 1.18e^{-0.0097A}, \qquad 2)$$

where

C = Specific activity (per cent of dose per Gm. of Ca), and

A = Calcium content of hard tissue (mg. of Ca per Gm. of tissue).

This relationship is significant ($p = 0.02$) and is a measure of calcification in this individual. Engfeldt, Engström, and Zetterström (31) have hypothesized a similar relationship for the uptake of P-32, although their diagram does not indicate

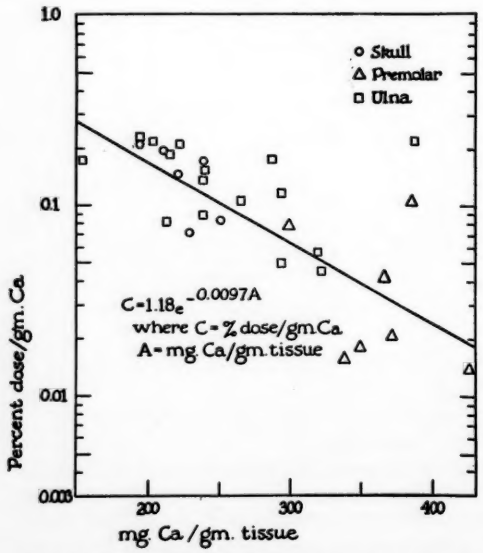

FIG. 4. RELATIONSHIP BETWEEN SPECIFIC ACTIVITY AND CALCIUM CONTENT OF BONES AND TEETH IN A PATIENT WITH GARGOYLISM 16 DAYS FOLLOWING THE INTRAVENOUS ADMINISTRATION OF 80 μC. OF $CA\text{-}45Cl_2$

[6] Mitchell, Hamilton, Steggerda, and Bean (39) estimate the Ca content of a normal boy weighing 16.4 Kg. as 185 Gm., and that of a normal 10 year old boy as 396 Gm. We have assumed our patient's body calcium content lay between these limits.

the exponential nature of the relationship. Plumlee, Hansard, Comar, and Beeson (41) have also reported an inverse relation between specific activity of calcium in bones of the bovine fetus and fetal age, but again not as an exponential function. Rogers, Weidmann, and Jones (33) have discussed the difficulty of interpreting these observations from the point of view of mechanisms.

On the whole there is good agreement between the data in Tables II and III, even though the size of the sample was very small in the case of the analyses reported in Table II; this shows that the sampling of bone with a dental burr at well-defined anatomical points can provide both accuracy and definition (23). This method should prove very helpful as a quantitative adjunct to autoradiography.

The findings listed in Tables II and III confirm the observations of Sognnaes, Shaw, and Bogoroch (42) that the teeth do not participate in the general metabolism of calcium. Thus, unerupted teeth showed some labeling, although much less than skeletal structures, while deciduous teeth had almost negligible radioactivity. The two deciduous canine teeth analyzed *in toto* averaged 168 mg. of calcium. If it is assumed that the calcium content of this patient's body was 300 Gm.,[6] then the canine teeth could have contained nearly 0.168 times 100 over 300 or 0.06 per cent of the labeled calcium when, in fact, they contained one-tenth this quantity.

In the case of one complete metacarpal bone removed at autopsy, a similar calculation would have led to an expected Ca-45 content of 0.01 per cent. This value was found experimentally and indicates high metabolic activity. If ossification was delayed in this as compared to other bones [ossification is often delayed in the metacarpal bone centers of patients with gargoylism (13)], one might expect a relatively higher calcification rate leading to greater concentration of the isotope. In addition, the calcium content of the metacarpal bone as a whole was only 75.6 mg. of Ca per Gm. as contrasted with the higher Ca content of the bone scrapings (*cf.*, Figure 4).

The analysis of the calvarium (Tables II and III) indicates that calcification was not primarily the cause of its immense thickening. The level of the specific activity of the exterior of the calvarium was low, approximating that of the teeth; at the interior, the calvarium had a specific activity typical of most bony tissues. Its rate of calcification was similar to that of the other hard tissues (Figure 4). Anderson, Emery, McAlister, and Osborn (10) have recently reported that the Ca-45 content of the skull of a myelomatous patient was 0.0096 μc. per Gm. or 0.00062 per cent dose per Gm. 60 days after administration of the isotope. Our patient's skull contained 0.0313 per cent dose per Gm. 15 days after administration of the Ca-45. Even if his level had dropped by half at the end of 60 days, our patient's skull would still have contained over 25 times more isotope than reported for the skull of the myelomatous adult.

Because Anderson and collaborators (10) reported that the Ca-45 tended to concentrate in the myelomatous regions, it is difficult to know whether the higher isotope level of the skull of our patient with gargoylism was the result of abnormal metabolic activity or whether the lower level in the myelomatous skull was due to preferential deposition in the myelomatous regions.

SUMMARY AND CONCLUSIONS

1. The rate of disappearance of Ca-45 from the serum of a 10 year old patient with terminal gargoylism is described by the following equation:

$$C_t = 0.177e^{-8.1t} + 0.061e^{-0.322t} + 0.035e^{-0.034t} + 0.018e^{-0.006t},$$

where

C_t = Specific activity of the serum (per cent of dose per mg. of Ca), and

t = Time (hours).

The exponents in the second and third terms of this equation, valid for 15 days, indicate an appreciably slower rate of disappearance than observed heretofore in normal boys and one young adult. The exponent in the fourth term indicates a rate comparable to that of normal adults, but slower than seen in normal boys.

2. The urinary calcium output of this patient was severely depressed, so that the urinary-fecal partition ratio of Ca-45 was 0.24, as contrasted with a more normal range of 1 to 2. The specific activities of serum and urine were found to be alike.

3. Fecal output of calcium and Ca-45 was in the normal range. The endogenous fecal output of this patient was calculated to equal 83 mg. per day, or 11.0 per cent of his average total excretion of calcium in the feces. This value is similar to that observed in other humans.

4. The depressed rate of disappearance of the injected Ca-45 from the blood is consistent with the possibility of decreased calcium deposition. The accretion value A, 0.54 Gm. of Ca per day, was much lower than that calculated for normal boys. The exchangeable fraction of body calcium, E, was 0.93 per cent of his estimated body calcium content of 300 Gm. This value is comparable to the E value of more normal subjects.

5. Data on the level of tracer in the soft tissues (brain, liver, spleen, gastrointestinal tract) obtained at necropsy 16 days after the start of the study were consistent with similar data in animals.

6. The level of Ca-45 in the mineralized tissues varied inversely and exponentially with the level of calcium in bone and tooth samples obtained with a dental burr at anatomically defined loci. This indicates that the labeling of calcified tissues corresponded to the rate of calcium deposition. Analyses of gross bone specimens supported this conclusion.

7. In the absence of comparable data on normal humans, it is difficult to know whether and how gargoylism caused the abnormal calcium metabolism of our patient.

ACKNOWLEDGMENTS

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REFERENCES

1. Geissberger, W. Die Calciumresorption und Retention beim Menschen nach intravenöser, oraler und rectaler Calciumverabreichung mit Bilanzen unter Anwendung von radioaktivem Calcium. Z. ges. exp. Med. 1952, **119**, 111.
2. Bellin, J., and Laszlo, D. Metabolism and removal of Ca-45 in man. Science 1952, **117**, 331.
3. Bronner, F., Harris, R. S., Maletskos, C. J., and Benda, C. E. Studies in calcium metabolism. Effect of food phytates on calcium-45 uptake in children on low-calcium breakfasts. J. Nutr. 1954, **54**, 523.
4. Blau, M., Spencer, H., Swernov, J., and Laszlo, D. Utilization and intestinal excretion of calcium in man. Science 1954, **120**, 1029.
5. Bronner, F., Harris, R. S., Maletskos, C. J., and Benda, C. E. Studies in calcium metabolism. The fate of intravenously injected radiocalcium in human beings. J. clin. Invest. 1956, **35**, 78.
6. Bronner, F., Harris, R. S., Maletskos, C. J., and Benda, C. E. Studies in calcium metabolism. Effect of food phytates on calcium-45 uptake in boys on a moderate calcium breakfast. J. Nutr. 1956, **59**, 393.
7. Bronner, F., and Harris, R. S. Absorption and metabolism of calcium in human beings, studied with calcium-45 *in* Ann. N. Y. Acad. Sci. 1956, **64**, 314.
8. Krane, S. M., Brownell, G. L., Stanbury, J. B., and Corrigan, H. The effect of thyroid disease on calcium metabolism in man. J. clin. Invest. 1956, **35**, 874.
9. Comar, C. L., and Wasserman, R. H. Radioisotopes in the study of mineral metabolism *in* Progress in Nuclear Energy, Series VI. Biological Sciences, J. C. Bugher, J. Coorsaget, and J. Loutit, Eds. New York, McGraw-Hill, 1956, p. 153.
10. Anderson, J., Emery, E. W., McAlister, J. M., and Osborn, S. B. The metabolism of a therapeutic dose of 45Ca in a case of multiple myeloma. Clin. Sci. 1956, **15**, 567.
11. Blau, M., Spencer, H., Swernov, J., Greenberg, J., and Laszlo, D. Effect of intake level on the utilization and intestinal excretion of calcium in man. J. Nutr. 1957, **61**, 507.
12. Spencer, H., Laszlo, D., and Brothers, M. Strontium^{85} and calcium^{45} metabolism in man. J. clin. Invest. 1957, **36**, 680.
13. Benda, C. E. Developmental Disorders of Mentation and Cerebral Palsies. New York, Grune and Stratton, 1952.
14. Lindsay, S., Reilly, W. A., Gotham, T. J., and Skahen, R. Gargoylism. II. Study of pathologic lesions and clinical review of twelve cases. Amer. J. Dis. Child. 1948, **76**, 239.
15. Henderson, J. L., MacGregor, A. R., Thannhauser, S. J., and Holden, R. The pathology and biochemistry of gargoylism. A report of three cases with a review of the literature. Arch. Dis. Childh. 1952, **27**, 230.
16. Brante, G. Gargoylism: a mucopolysaccharidosis. Scand. J. clin. Lab. Invest. 1952, **4**, 43.
17. Dawson, I. M. P. The histology and histochemistry of gargoylism. J. Path. Bact. 1954, **67**, 587.
18. Uzman, L. L. Chemical nature of the storage substance in gargoylism. Arch. Path. 1955, **60**, 308.
19. Bishton, R. L., Norman, R. M., and Tingey, A. The pathology and chemistry of a case of gargoylism. J. clin. Path. 1956, **9**, 305.

20. Stacey, M., and Barker, S. A. Chemical analysis of tissue polysaccharides. J. clin. Path. 1956, **9**, 314.
21. Amprino, R. Uptake of ^{35}S in the differentiation and growth of cartilage and bone *in* Ciba Foundation Symposium on Bone Structure and Metabolism, G. E. W. Wolstenholme and C. M. O'Connor, Eds. Boston, Little, Brown and Co., 1956, p. 89.
22. Sognnaes, R. F., and Volker, J. F. Studies on the distribution of radioactive phosphorus in the tooth enamel of experimental animals. Amer. J. Physiol. 1941, **133**, 112.
23. Sognnaes, R. F., and Shaw, J. H. Salivary and pulpal contributions to the radiophosphorus uptake in enamel and dentin. J. Amer. dent. Ass. 1952, **44**, 489.
24. Salomon, K., Gabrio, B. W., and Smith, G. F. A precision method for the quantitative determination of calcium in blood plasma. Arch. Biochem. 1946, **11**, 433.
25. Greenberg, D. M., and Troescher, F. M. Study with radioactive isotopes of excretion of calcium and strontium by way of the bile. Proc. Soc. exp. Biol. (N. Y.) 1942, **49**, 488.
26. Singer, L., Maqsood, M., Medlen, A. B., and Comar, C. L. Endogenous and biliary excretion of calcium-45 and strontium-89. Arch. Biochem. Biophys. 1957, **66**, 404.
27. Bronner, F. Disposition of intraperitoneally injected calcium-45 in suckling rats. J. gen. Physiol. 1958. In press.
28. Hevesy, G. Ch., Levi, H. B., and Rebbe, O. H. Rate of rejuvenation of the skeleton. Biochem. J. 1940, **34**, 532.
29. Amprino, R. Autoradiographic analysis of the distribution of labelled Ca and P in bone. Experientia 1952, **8**, 20.
30. Amprino, R. Further experiments on the fixation *in vitro* of radiocalcium to sections of bone. Experientia 1952, **8**, 380.
31. Engfeldt, B., Engström, A., and Zetterström, R. Renewal of phosphate in bone minerals. II. Radioautographic studies of the renewal of phosphate in different structures of bone. Biochim. biophys. Acta 1952, **8**, 375.
32. Lacroix, P., Devis, R., and Schicks, E. Distribution of radiophosphorus in the long bones of adult rabbits. Experientia 1952, **8**, 113.
33. Rogers, H. J., Weidmann, S. M., and Jones, H. G. Studies on the skeletal tissues. 3. The rate of exchange of the inorganic phosphate in different bones and parts of bones in various species of mammal. Biochem. J. 1953, **54**, 37.
34. Engfeldt, B., and Hjertquist, S. O. Biophysical studies on bone tissue. X. The *in vivo* and *in vitro* uptake of radioactive isotopes and ionic exchange reactions in bone tissue. Acta path. microbiol. scand. 1954, **35**, 205.
35. Vincent, J. Recherches sur la constitution de l'os adulte. Thesis, Faculty of Medicine, Université Catholique de Louvain, 1955.
36. Weidmann, S. M. Studies on the skeletal tissues. 4. The renewal of inorganic phosphate in bones of various species of small mammal as a function of time. Biochem. J. 1956, **62**, 593.
37. Kulp, J. L., Eckelmann, W. R., and Schulert, A. R. Strontium-90 in man. Science 1957, **125**, 219.
38. Bauer, G. C. H., Carlsson, A., and Lindquist, B. Evaluation of accretion, resorption, and exchange reactions in the skeleton. Kungl. Fysiograf. Sällsk., Lund, Förhandl. 1955, **25**, 1.
39. Mitchell, H. H., Hamilton, T. S., Steggerda, F. R., and Bean, H. W. The chemical composition of the adult human body and its bearing on the biochemistry of growth. J. biol. Chem. 1945, **158**, 625.
40. Dziewiatkowski, D. D., Bronner, F., Di Ferrante, N., and Archibald, R. M. Some aspects of the metabolism of sulfate-S-35 and calcium-45 in the metaphyses of immature rats. Influence of β-estradiol benzoate. J. biophys. biochem. Cytol. 1957, **3**, 151.
41. Plumlee, M. P., Hansard, S. L., Comar, C. L., and Beeson, W. M. Placental transfer and deposition of labeled calcium in the developing bovine fetus. Amer. J. Physiol. 1952, **171**, 678.
42. Sognnaes, R. F., Shaw, J. H., and Bogoroch, R. Radiotracer studies on bone, cementum, dentin and enamel of rhesus monkeys. Amer. J. Physiol. 1955, **180**, 408.

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DEMONSTRATION OF A CIRCULATING ANTICOAGULANT IN PLASMA THROMBOPLASTIN ANTECEDENT DEFICIENCY

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(Submitted for publication July 23, 1957; accepted September 12, 1957)

Resistance to transfusion therapy in the hereditary hemorrhagic syndromes has long been known to occur. Lawrence and Johnson (1) in 1941 demonstrated a circulating anticoagulant in a case of classical hemophilia. Numerous reviews on the subject of circulating anticoagulants have recently appeared (2–4). Richards and Spaet (5), and Lewis and Didisheim (6) have been able to produce an antibody directed against antihemophilic globulin (AHG) in animals. These studies give support to the concept of an immunologic mechanism for the production of a circulating anticoagulant in hemophilics. Resistance to transfusion in plasma thromboplastin component deficiency (PTC) has also been reported (3, 7).

Recently the opportunity arose to study a resistant state in a case of plasma thromboplastin antecedent deficiency (PTA). This case has previously been reported as a demonstration of the steps necessary to correct the hemorrhagic diathesis before and during surgery (8). The usual techniques for demonstration of a circulating anticoagulant were uniformly unsuccessful in this patient. A modification of the thromboplastin generation test (TGT) was used which enabled us to demonstrate the resistant state *in vitro*.

METHODS

Whole blood clotting time was done by the Lee-White method (9). One stage plasma prothrombin time was performed according to Quick's technique (10), and serum prothrombin time was done by a modification of Quick's method (11). The thromboplastin generation test was done as described by Biggs and Douglas (12). The search for circulating anticoagulants was performed by mixing normal and patient's blood and determining the clotting times (13) and serum prothrombin times of such mixtures (4). Plasma recalcification times of similar mixtures were also performed (3). Heparin protamine titration test was done following the technique of Allen and co-workers (14).

Case presentation. This case has previously been reported as one of a family demonstrating PTA deficiency (see Table I and Figure 1) (15). Patient I. S. is a 50 year old white female. In 1943, 1947, and 1948 she required surgery for various diseases. Each surgical episode necessitated a large number of blood transfusions for correction of operative hemorrhage. In 1955 she was examined in this department and was found to have PTA deficiency.

In August of 1956 she was again admitted to Michael Reese Hospital for removal of a stone in the cystic duct. At this time it was noted that she had a marked resistance to transfusion, and correction of her hemorrhagic diathesis came about only by the use of a modified exchange transfusion procedure. At this time demonstration of a circulating anticoagulant by the usual techniques was unrewarding (see Table II). Three months after surgery further studies were undertaken to define the state of resistance in this patient.

Special techniques. At the present time the available evidence indicates that the state of resistance in the various hereditary hemorrhagic deficiencies is most likely due to the development of an antibody to the coagulation factor which is lacking. This resistance presumably comes about because of immunization by transfusion of normal blood or plasma. This so-called antibody is usually demonstrated by the anticoagulant effect on normal blood. Some antigen-antibody reactions are demonstrable only by special techniques, one of the most common being incubation at 37° C. We, therefore, undertook to perform the thromboplastin generation test, using mixtures of one part of the patient's reagent to nine parts of the normal reagent. Plasma recalcification times were performed in the same manner. All mixtures and individual reagents were incubated at 37° C. for four hours. The reagents were tested to determine loss of potency and none was found. Both normal and the patient's substrates were used and no difference was found. Four hours was chosen for the incubation time because of technical reasons in doing such a large number of generation studies.

RESULTS

In the technique of plasma recalcification time, mixtures of one part patient's plasma to nine parts normal plasma were incubated at 37° C. for four hours. At half-hourly intervals, tubes were removed and the recalcification time determined. A second series using patient's adsorbed plasma was also examined. Incubation of these mixtures

TABLE I

Proof of PTA deficiency on basis of crossmatching experiments

Mixture*	Clotting time (*minutes*)	Serum prothrombin time (*seconds*)
Without any addition	45	11
Addition of:		
NaCl	41	12
Normal $BaSO_4$ plasma	7	60
Hemophilic $BaSO_4$ plasma	7	40
Hageman factor $BaSO_4$ plasma	8½	35
Normal aged serum	6½	39
PTC deficient serum	8	56
Hageman factor deficient serum	10	32
PTA deficient serum	39	11
PTA deficient plasma	41	11

* All addition experiments were performed by adding 0.1 ml. of the reagent to 0.9 ml. of the tested blood.

did not provide any evidence of inhibition of normal plasma. The use of a 50 per cent mixture of plasmas also showed no effect of an anticoagulant on the normal plasma. On the contrary, these

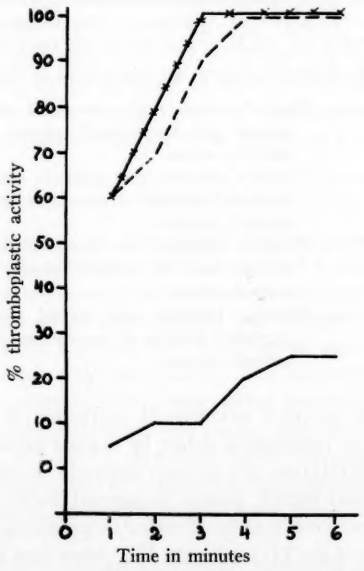

FIG. 1. THROMBOPLASTIN GENERATION TEST

The different mixtures are represented as follows:

——— Patient's platelets plus patient's $BaSO_4$ plasma plus the patient's serum.

—x—x— Patient's platelets plus normal $BaSO_4$ plasma plus the patient's serum.

- - - - - Patient's platelets plus patient's $BaSO_4$ plasma plus the normal serum.

TABLE II

Tests for circulating anticoagulants

Mixture	Type of test: Clotting time (*minutes*)	Type of test: Serum prothrombin time (*seconds*)
1 ml. normal blood	9.6	
1 ml. normal blood + 0.3 ml. NaCl	10	
1 ml. normal blood + 0.3 ml. patient's blood	10	
2 ml. normal blood		35
2 ml. normal blood + 0.3 ml. NaCl		33
2 ml. normal blood + 0.3 ml. patient's blood		29

	Plasma recalcification time* (*seconds*)	Incubated† plasma recalcification time (*seconds*)
0.2 ml. normal plasma	120	180
0.1 ml. normal plasma + 0.1 ml. patient's plasma	120	195
0.2 ml. patient's plasma	150	185
0.1 ml. patient's $BaSO_4$ plasma + 0.1 ml. normal plasma	125	190

* Plasma recalcification times performed by mixing equal volumes of plasma and 0.2 M $CaCl_2$.

† Incubation carried out at 37° C. for four hours.

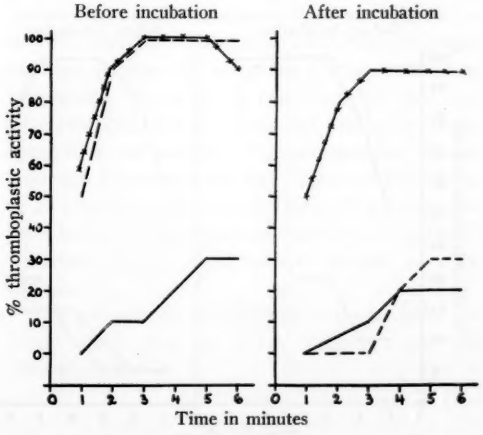

FIG. 2. THROMBOPLASTIN GENERATION TEST USING A MIXTURE OF NORMAL AND THE PATIENT'S ADSORBED PLASMA

The different mixtures are represented as follows:

——— Patient's platelets plus patient's $BaSO_4$ plasma plus the patient's serum.

—x—x— Patient's platelets plus normal $BaSO_4$ plasma plus the patient's serum.

- - - - - Patient's platelets plus mixture of normal plus patient's $BaSO_4$ plasma plus the patient's serum.

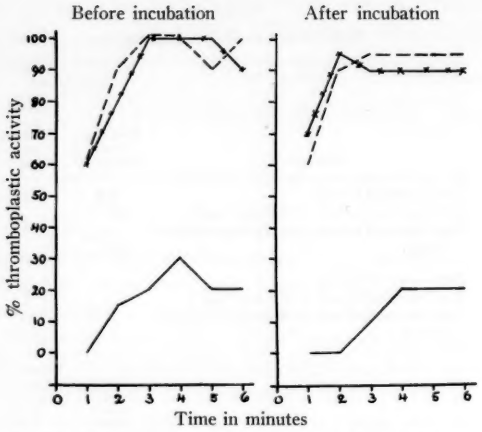

FIG. 3. THROMBOPLASTIN GENERATION TEST USING A MIXTURE OF NORMAL AND THE PATIENT'S SERUM

The different mixtures are represented as follows:

——— Patient's platelets plus patient's $BaSO_4$ plasma plus the patient's serum.

- - - - - Patient's platelets plus patient's $BaSO_4$ plasma plus the normal serum.

—x—x— Patient's platelets plus patient's $BaSO_4$ plasma plus mixture of normal and the patient's serum.

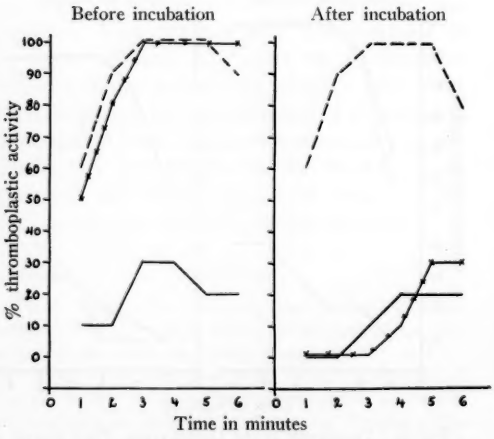

FIG. 4. THROMBOPLASTIN GENERATION TEST USING MIXTURES OF PATIENT'S ADSORBED PLASMA AND NORMAL SERUM

The different mixtures are represented as follows:

——— Patient's platelets plus patient's $BaSO_4$ plasma plus the patient's serum.

- - - - - Patient's platelets plus patient's $BaSO_4$ plasma plus the normal serum.

—x—x— Patient's platelets plus patient's $BaSO_4$ plasma plus mixture of normal serum and the patient's $BaSO_4$ plasma.

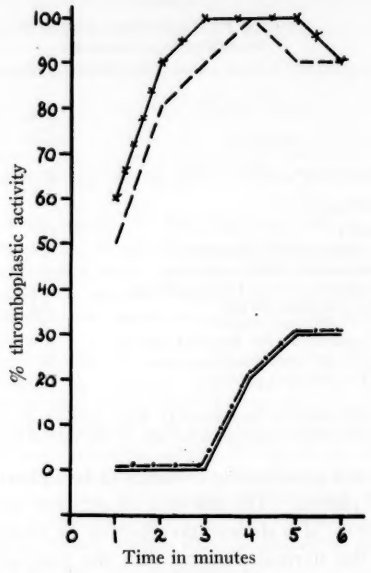

FIG. 5. THROMBOPLASTIN GENERATION TEST DEMONSTRATING EFFECT OF ADDITION OF NONADSORBED PLASMA OR SERUM ON THE MIXTURES INCUBATED FOR FOUR HOURS

The different mixtures are represented as follows:

——— Patient's platelets plus incubated mixture of normal and the patient's plasma plus the patient's serum.

—·—·— Patient's platelets plus patient's plasma plus incubated mixture of normal serum and the patient's plasma.

- - - - - Patient's platelets plus incubated mixture of normal and the patient's plasma plus the normal serum.

—x—x— Patient's platelets plus normal plasma plus incubated mixture of normal serum and the patient's plasma.

tests provided evidence of correction of the patient's coagulation defect by normal plasma.

A mixture of one part patient's to nine parts normal $BaSO_4$ plasma or normal $BaSO_4$ plasma alone were equally effective in producing correction of the TGT (Figure 1). After four hours incubation, the mixture had lost its potency; however, the incubated non-mixed normal $BaSO_4$ plasma was as effective as before (Figure 2).

Figure 3 demonstrates the results using mixtures of normal and the patient's serum. No loss of thromboplastic activity is evident after incubation.

Figure 4 shows that a mixture of normal serum

and patient's adsorbed plasma resulted in a marked loss of thromboplastic activity after incubation.

Figure 5 demonstrates an experiment designed to rule out any inhibitory effect of the anticoagulant against either AHG or PTC. When the patient's and normal $BaSO_4$ plasma were incubated for four hours, correction in the TGT could be accomplished by addition of normal serum (which does not contain AHG). This then rules out AHG inhibition. In like manner, when a four hour incubation of the patient's plasma and normal serum was tested against normal plasma, correction was again achieved indicating that PTC was not inhibited (normal adsorbed plasma does not contain PTC). Correction by addition of either the patient's plasma or serum could not be obtained.

Precipitation of the anticoagulant from the plasma was accomplished by ammonium sulfate fractionation. A 25 to 33 per cent ammonium sulfate solution produced complete removal of the anticoagulant. Zero to 20 per cent saturation removed only small amounts of the anticoagulant. Rosenthal (16) found maximal activity of the PTA substance in the 25 to 33 per cent fraction. Heating of the patient's plasma for 20 minutes at 60° C. removed all traces of anticoagulant activity as did storage for three months at 4° C.

DISCUSSION

Analysis of the data reveals that the patient under study demonstrated a primary PTA deficiency with a previously undescribed circulating anticoagulant. This anticoagulant was present only in the patient's plasma and was directed against the formation of thromboplastin. The inhibitory substance acted against a coagulation factor which is present in both serum and plasma and was neither AHG nor PTC. Previous studies revealed that our patient lacked only PTA and therefore the circulating anticoagulant was most likely directed against PTA.

These results are not entirely surprising because it is well known that individuals with AHG or PTC deficiency have been reported to develop circulating anticoagulants following transfusion. The finding of an inhibitor of PTA in a known case of PTA deficiency was therefore predictable. Our lack of knowledge of the patient's status prior to transfusion does not allow us to state with certainty that we are dealing with an acquired form of circulating anticoagulant. We have been unable to obtain other known PTA deficient members of this patient's family for similar studies. Experience with the resistant state in PTC or AHG deficiency would lead us to conclude that most likely this is an acquired circulating anticoagulant.

It is of interest that the patient's serum did not possess any anticoagulant activity. No reason is known for this; one can only speculate that the anticoagulant is either inactivated, or used up during the coagulation process. (It is possible that treatment of the serum with $BaSO_4$ might have brought out the activity of the anticoagulant; however, we have been unable to have the patient return for these studies.)

A heparin-like anticoagulant is ruled out on the basis of a normal protamine titration test and because no abnormalities of the plasma prothrombin time were ever elicited.

Of major interest in this study is the demonstration of a circulating anticoagulant by the use of an incubation technique in the thromboplastin generation test. Bergna and Pavlovsky (7) have shown that small amounts of time may elapse before an inhibitory effect of a circulating anticoagulant can be detected using the plasma recalcification time. No effect on recalcification time was demonstrable after four hours of incubation of reagent from our patient. The more sensitive thromboplastin generation test gave substantial evidence of an inhibitory effect of our patient's plasma after four hours of incubation of mixtures of patient's absorbed plasma with either normal sera or plasma.

The patient was reexamined one year after the initial study and the PTA inhibitor was still demonstrable.

SUMMARY

The demonstration of a circulating anticoagulant in PTA deficiency is described for the first time.

The anticoagulant could only be demonstrated by the use of the thromboplastin generation test after incubation of the patient's plasma with either normal sera or plasma.

The anticoagulant activity is confined to the patient's plasma.

REFERENCES

1. Lawrence, J. S., and Johnson, J. B. The presence of a circulating anticoagulant in a male member of a hemophiliac family. Trans. Amer. clin. climat. Ass. 1941, **57**, 223.
2. Margolius, A., Jr., and Ratnoff, O. D. A laboratory study of the carrier state in classic hemophilia. J. clin. Invest. 1956, **35**, 1316.
3. Lewis, J. H., Ferguson, J. H., and Arends, T. Hemorrhagic disease with circulating inhibitors of blood clotting: Anti-AHF and anti-PTC in eight cases. Blood 1956, **11**, 846.
4. Stefanini, M., and Dameshek, W. The Hemorrhagic Disorders. New York, Grune & Stratton, 1955.
5. Richards, M. D., and Spaet, T. H. Immunization of rabbits against human antihemophilic factor (AHF). Blood 1956, **11**, 473.
6. Lewis, J. H., and Didisheim, P. Production of anti-human PTC and anti-human proconvertin in rabbits. Proc. Soc. exp. Biol. (N. Y.) 1956, **93**, 429.
7. Bergna, L. J., and Pavlovsky, A. Classification of hemophiliacs with development of an anticoagulant. Acta haemat. 1956, **16**, 247.
8. Lisker, R., Josephson, A. M., Werbin, G., Shapiro, C. M., and Rozengvaig, S. The correction of a hemorrhagic diathesis in preparation for surgery: The correction of plasma thromboplastin antecedent deficiency. Arch. intern. Med. 1957, **100**, 474.
9. Lee, R. I., and White, P. D. A clinical study of the coagulation time of blood. Amer. J. med. Sci. 1913, **145**, 495.
10. Quick, A. J. The Hemorrhagic Diseases and the Physiology of Hemostasis. Springfield, Charles C Thomas, 1942.
11. Quick, A. J. The coagulation mechanism with specific reference to the interpretation of prothrombin time and a consideration of the prothrombin consumption time. Amer. J. clin. Path. 1949, **19**, 1016.
12. Biggs, R., and Douglas, A. S. The thromboplastin generation test. J. clin. Path. 1953, **6**, 23.
13. Ramot, B., and Singer, K. An unusual circulating anticoagulant in systemic lupus erythematosus. Acta haemat. 1956, **16**, 158.
14. Allen, J. G., Moulder, P. V., Elghammer, R. M., Grossman, B. J., McKeen, C. L., Sanderson, M., Egner, W., and Crosbie, J. M. A protamine titration as an indication of a clotting defect in certain hemorrhagic states. J. Lab. clin. Med. 1949, **34**, 473.
15. Ramot, B., Angelopoulos, B., and Singer, K. Plasma thromboplastin antecedent deficiency. Arch. intern. Med. 1955, **95**, 705.
16. Rosenthal, R. L. Properties of plasma thromboplastin antecedent (PTA) in relation to blood coagulation. J. Lab. clin. Med. 1955, **45**, 123.

ANNOUNCEMENTS OF MEETINGS

The Fiftieth Annual Meeting of THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION will be held in Atlantic City, N. J., on Monday, May 5, 1958, at 9:00 A.M. at the Casino Theater on the Steel Pier.

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THYROXINE-BINDING BY SERUM PROTEIN IN PREGNANCY AND IN THE NEWBORN

By JACOB ROBBINS and JAMES H. NELSON

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(Submitted for publication August 31, 1957; accepted September 26, 1957)

Thyroid structure and function in man undergo a number of changes during pregnancy. The thyroid gland may become enlarged and hyperplastic (1, 2), thyroidal uptake of radioiodine is increased (3, 4), "conversion" of blood iodide to organic form is more rapid (4), and the serum protein-bound iodine (PBI) is elevated (5, 6). Although these alterations are like those seen in hyperthyroidism, they appear to be associated, in early pregnancy at least, with an unequivocally euthyroid state. Whereas the PBI rises as early as the first or second month of gestation, and remains at about the same level throughout pregnancy, elevation of the basal metabolic rate (2, 7) begins in the latter half of pregnancy and rises progressively. Circulatory changes, such as increased cardiac output (8), may precede the rise in oxygen consumption but these also change progessively until shortly before term.

It has been postulated that alterations in the interaction of thyroid hormone with serum proteins might provide an explanation for these paradoxical findings (6). Inasmuch as circulating thyroxine is largely bound to what appears to be a specific alpha globulin (TBP) (9, 10), attention has been directed toward this protein in pregnancy. It has been demonstrated that TBP in pregnancy has an increased ability to complex thyroxine (10–13). In the present study, the thyroxine-binding capacity of TBP has been quantitated during the course of normal pregnancy and in newborn infants. From these data it has been possible to calculate an approximate concentration of free thyroxine in the serum of these subjects.

METHODS

Serum was obtained from 16 women during and after uncomplicated pregnancies, usually after an overnight fast, from umbilical vein blood sampled at delivery in three subjects, and from external jugular venipuncture in two infants on the third day after delivery. Mixtures of serum with I^{131}-labeled L-thyroxine [1] were prepared according to the procedure described previously (14). Prior to use, each of the eight thyroxine preparations employed was tested for radiochemical purity by chromatography, and was repurified, when necessary, to a purity of 90 per cent or better by an extraction procedure (15). Iodine analyses [2] were performed by a modification of the method of Zak, Willard, Myers, and Boyle (16). The thyroxine-serum mixtures were subjected to zone electrophoresis on filter paper strips, using barbital buffer, pH 8.6, ionic strength 0.1, and a "reverse-flow" technique (15). This modification of the electrophoresis procedure avoided the inclusion of albumin-bound thyroxine in the alpha globulin zone.

For measurement of the thyroxine-binding capacity of TBP, the quantity of thyroxine added to each serum was sufficient to effectively saturate the thyroxine-binding sites on TBP. Actual saturation of these sites is approached asymptotically (15). Analysis of data obtained with normal sera (10), however, had indicated that the amount of thyroxine associated with alpha globulin reaches an effective plateau when less than 60 per cent of the serum thyroxine is in the alpha globulin zone. In most instances, the mixtures were such that 20 per cent to 40 per cent of the thyroxine were in the alpha globulin zone. This was usually achieved by the addition of between 1 and 2 μg. of thyroxine per ml. of serum. The average value for at least three serum mixtures, expressed as the quantity of thyroxine associated with TBP, was taken as the thyroxine-binding capacity of TBP. Barring any qualitative variation in the TBP sites for thyroxine, this value expressed in moles would be equivalent to the concentration of such sites.

Equations for the calculation of free or unbound thyroxine in serum have been described earlier (10). In the present calculations, the values employed for serum albumin concentration were 4.5 Gm. per cent in nonpregnant adults, and 3.4 Gm. per cent in pregnancy and in the newborn (17). The basis for the use of assumed rather than measured albumin values, and other assumptions involved in the calculations are considered in the discussion.

[1] Obtained from Abbott Laboratories, Oak Ridge, Tennessee.

[2] Performed by J. Benotti, Boston Medical Laboratories, Boston, Massachusetts.

TABLE I

Thyroxine and TBP in serum during normal pregnancy, in the newborn, and in nonpregnant adults

	No. of samples	Total thyroxine*			TBP sites for thyroxine†			Free thyroxine		
		Range	Mean ± S.D.	p‡	Range	Mean ± S.D.	p‡	Range	Mean ± S.D.	p‡
		($\times 10^{-7}$ M)§			($\times 10^{-7}$ M)			($\times 10^{-11}$ M)		
Pregnancy										
First trimester	6	1.3–2.1	1.6 ± 0.3	<0.001	4.5–8.0	6.2 ± 1.1	<0.001	3.5–5.9	4.5 ± 1.0	0.05
Second trimester	10	1.0–1.9	1.5 ± 0.3	<0.001	5.3–9.3	7.2 ± 1.3	<0.001	2.9–3.9	3.3 ± 0.4	<0.001
Third trimester	20	1.0–2.2	1.6 ± 0.3	<0.001	4.0–10.7	6.4 ± 1.7	<0.001	2.3–6.6	4.2 ± 1.1	<0.001
Six wks. post partum	5	0.55–1.2	0.92 ± 0.25	>0.8	2.1–3.5	2.8 ± 0.6	>0.4	4.0–6.2	5.3 ± 0.9	>0.3
Newborn	5	1.2–1.9	1.5 ± 0.3	<0.001	3.0–4.9	4.3 ± 0.7	<0.001	4.3–9.4	6.9 ± 2.2	>0.5
Nonpregnant adults‖	9	0.71–1.1	0.95 ± 0.14		2.1–3.2	2.6 ± 0.4		3.3–8.6	6.2 ± 1.7	

* Assuming that all of the serum PBI is thyroxine.
† Thyroxine-binding capacity of TBP.
‡ Significance of the difference between the mean for each group and the mean for nonpregnant adults (19).
§ (Moles thyroxine per liter) $\times$ (7.77×10^6) = (μg. thyroxine per ml.); (μg. thyroxine per ml.) $\times$ 65.5 = (μg. thyroxine-iodine per 100 ml.).
‖ Further details on the nonpregnant adults have been presented elsewhere (10, 15). The subjects included five males and four females, there being no significant difference between the sexes with respect to thyroxine-binding.

RESULTS

From a qualitative standpoint, no differences were detected between thyroxine-protein interactions in serum from normal adult, pregnant, and newborn subjects. At concentrations of the order of 0.2 μg. or less of added thyroxine per ml. of serum, most of the serum radioiodine had an electrophoretic mobility, at pH 8.6, intermediate between alpha-1 and alpha-2 globulin. The remainder was associated with albumin, with the exception of approximately 5 per cent or less which was sometimes found just ahead of albumin. This "prealbumin" zone was poorly separated from albumin, and its significance is not clear (18). The beta and gamma globulin zones did not contain significant amounts of thyroxine. When larger amounts of thyroxine were added to the serum, a point was reached at which the thyroxine associated with alpha globulin no longer increased. Excess thyroxine migrated with albumin.

Results of quantitative studies in normal pregnancy are summarized in Table I. Included are multiple samples from five subjects who were followed throughout pregnancy (*cf.*, Table II), samples from two subjects studied only in the second month of gestation, and samples from nine subjects studied only at term. The results are expressed as molar concentrations in order to permit interchangeable use of the terms "thyroxine-binding capacity of TBP" and "concentration of TBP sites for thyroxine." Factors for conversion of moles to the more familiar μg. of thyroxine and μg. of protein-bound iodine are given in Table I.

Total serum thyroxine was elevated above the normal range early in pregnancy (the earliest time studied was in the sixth week of gestation) and persisted without significant change until term. By six weeks post partum, the level was back to normal. The thyroxine-binding capacity of TBP was likewise elevated at the earliest time examined. The increase in thyroxine-binding capacity, however, was relatively greater than the rise in total thyroxine. As a consequence, from 15 to 34 per cent (mean, 24 per cent) of the available sites on TBP were occupied in pregnancy in contrast to from 21 to 42 per cent (mean, 34 per cent) in nonpregnant adults. The mean thyroxine-binding capacity for the various trimesters did not differ significantly from each other ($p > 0.1$) and, in the

TABLE II

Thyroxine and TBP in serum during normal pregnancy

	Total thyroxine ($\times 10^{-7}$ M)					TBP sites for thyroxine ($\times 10^{-7}$ M)					Free thyroxine ($\times 10^{-11}$ M)				
Case no.	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Months* pregnant															
2		1.3			1.8		4.5			8.0		4.7			3.5
3		1.4		2.1			6.1		6.2			3.7		5.9	
4	1.0	1.4	1.4			5.3	7.2	5.9			2.9	3.0	3.8		
5	1.4	1.4	1.4			5.8	7.0	7.5			3.9	3.1	2.9		
6		1.8	1.5	1.9	1.8		7.3	7.7	8.9	9.3		3.9	3.0	3.4	2.9
7	1.5		1.5	2.2		6.7		6.6	7.0		3.6		3.7	5.5	
8	1.8	1.5	1.8			7.6	6.7	5.7			3.8	3.6	5.3		
9	1.5			1.7		9.3			10.7		2.4			2.3	
Term		1.7	2.2		1.9		7.7	6.0		7.6		3.3	6.6		4.1
Days post partum															
3	1.5	1.7		1.8	1.4	7.0	7.6		7.7	8.4	3.4	3.4		3.7	2.5
10		1.5	1.5	1.5			6.3	4.8	6.6			3.9	5.5	3.7	
24				1.2					4.5					4.3	
31	1.1					3.9					4.7				
38			1.1					3.2					5.9		

* Lunar.

subjects studied serially (Table II), no consistent trend during pregnancy was observed.

The calculated free thyroxine, on the other hand, did not increase. Indeed, the mean value for each pregnancy group was significantly lower than in the nonpregnant subjects. The majority of the individual values in pregnancy, however, fell within the range found for nonpregnant subjects. When the trimesters of pregnancy were compared, the mean free thyroxine for the first and third trimesters were not significantly different ($p > 0.5$), whereas the mean for the second trimester was significantly lower ($p < 0.01$, $p < 0.02$). In the subjects in Table II, however, no consistent trend during pregnancy was observed, suggesting that this difference may be in error due to the small samples.

Both free thyroxine and the thyroxine-binding capacity returned to normal by the sixth week post partum, together with the PBI.

The findings in the newborn are presented in Table III and summarized in Table I. Total thyroxine was significantly higher than in nonpregnant adults, and was equal to or lower than in the mother. The thyroxine-binding capacity of TBP, although significantly higher than in nonpregnant

TABLE III

Thyroxine and TBP in serum of newborn

	Total thyroxine ($\times 10^{-7}$ M)					TBP sites for thyroxine ($\times 10^{-7}$ M)					Free thyroxine ($\times 10^{-11}$ M)				
Case no.	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Mother*	1.5	1.7	2.2	1.8	1.9	7.0	7.7	6.0	7.7	7.6	3.4	3.3	6.6	3.7	4.1
Fetus†		1.2	1.4		1.9§		4.5	4.5		4.4		4.3	5.3		8.7§
					1.3										7.2
Infant‡	1.4			1.8		3.0			4.9		9.4			6.7	

* Mother's serum obtained at same time as fetus or infant.
† Umbilical vein at term.
‡ Three days post partum.
§ The PBI in this serum was not measurable (< blank), possibly due to contamination with mercury. The infant appeared to be normal. Two values for total thyroxine were assumed—one equal to that in the mother, the other differing from the mother to the extent found in Case No. 3. The former value was used in Table I.

adults, was consistently lower than in the mother. Free thyroxine was, in all but one case, higher than in the mother, but not significantly different from the nonpregnant adult value.

DISCUSSION

It is clear from the present study, and from earlier evidence (10–13), that uncomplicated pregnancy in man is accompanied by an increase in thyroxine-binding by at least one component of the serum alpha globulins. This thyroxine-binding alpha globulin in pregnancy has the characteristic electrophoretic mobility of TBP in normal human serum both at pH 8.6 and at pH 4.5 (13). As in normal serum, there is no direct evidence that TBP is a single protein; nevertheless, this seems likely in view of the highly specific nature of its interaction with thyroxine (20, 21) and its very low concentration in serum (10). In the foregoing treatment of the data, and in this discussion, the assumption is made that TBP is a single protein species.

The increase in thyroxine-binding capacity of TBP, as measured in this study, could result either from an increase in the number or availability of binding sites per molecule of TBP, or from an increase in the concentration of TBP. The latter possibility appears more reasonable, although no evidence bears directly on this question. Other serum proteins undergo changes in concentration during pregnancy, and the alpha globulins, as a group, increase (22, 23). It is possible that TBP participates in these changes and that the alterations in thyroid physiology during pregnancy are a fortuitous result of this initial event. Since it has been shown that administration of estrogen to nonpregnant individuals results in an increase of PBI (24) and TBP (25, 26) in serum, it appears that the increase in estrogen which occurs during pregnancy (27) may initiate these changes in the pregnant subject.

The mechanism by which a change in the concentration of TBP might affect thyroid physiology is, likewise, a matter for speculation. Circumstantial evidence presented earlier (10, 28) has suggested a correlation between the level of free thyroxine in serum, as calculated by the methods described, and the physiological state of the individual with respect to hyper-, hypo-, or euthyroidism. Since an increase in TBP without a concomitant change in thyroxine would result in a low free thyroxine level, homeostatic mechanisms may operate in such a situation to restore the normal free thyroxine level. This could be achieved by an increase in hormone secretion by the thyroid gland, by a decrease in thyroxine degradation, or by a combination of these processes. Evidence quoted in the introduction indicates that thyroid gland function does, in fact, increase. Thyroxine degradation in human pregnancy has not been defined, but it has been suggested (10) that, at least in some situations, the amount of thyroxine degraded may be proportional to the free thyroxine level in serum. In the study by Danowski, Schwartz, Rhodes, Yoder, Puntereri, and Mateer (29), although no difference was observed between pregnant and nonpregnant subjects, the use of nonphysiological amounts of thyroxine makes interpretation difficult.

It is probably significant, however, that, despite evidence that thyroid gland hyperfunction persists throughout pregnancy (4), the levels of PBI, TBP and free thyroxine remain constant after the initial rise in the first trimester. This suggests that factors other than those discussed above may be operative; for example, a progressive increase in thyroxine degradation in pregnancy could explain this discrepancy. Such an increase might also be responsible for the failure of the mean free thyroxine level in pregnancy to equal that in nonpregnant adults. The slightly lower levels in pregnancy, however, appear sufficient to maintain a state of euthyroidism.

Several other observations are consistent with the postulated interrelationship between the concentration of TBP sites for thyroxine and the level of free thyroxine, and with the effect of the latter on the physiological state of the subject. The amelioration of hyperthyroidism which sometimes occurs during pregnancy (*e.g.*, 30) could result from an increase in TBP sites in the presence of unaltered thyroid hormone secretion. In hyperthyroidism *per se*, the concentration of TBP sites remains normal (10), but in pregnancy complicated by hyperthyroidism, the expected increase in TBP sites takes place (26). Estrogen treated subjects are able to tolerate PBI levels higher than normal without development of hyperthyroid signs (24, 25); and, in athyreotic subjects on constant thyroid hormone replacement therapy, es-

trogen administration may induce signs of hypothyroidism (25).

The observation has been made that I^{131}-labeled triiodothyronine passes less readily from pregnancy serum into red cells *in vitro* than from normal serum (31). This finding is also consistent with a low free thyroxine concentration and a relatively high concentration of unoccupied TBP sites, since triiodothyronine interacts with these TBP sites (10).

It is important to reemphasize (10), at this point, that a number of assumptions are involved in the calculation of free thyroxine as employed in this study. Included among these are the assumptions: *a*) that albumin and TBP each have a single type of binding site for thyroxine at the thyroxine levels employed, *b*) that there are no interactions between successively bound thyroxine molecules, and *c*) that zone electrophoresis provides satisfactory estimates of the maximum number of sites on TBP and of the ratio between the intrinsic association constants for the thyroxine-albumin and thyroxine-TBP complexes. These assumptions, as discussed elsewhere (10, 15), appear to be valid.

Other assumptions are: *d*) that the thyroxine-protein interaction *in vitro* (*i.e.*, in barbital buffer at pH 8.6 under equilibrium conditions) is the same as under physiological conditions *in vivo*, *e*) that human and bovine albumin do not differ in their interaction with thyroxine, and *f*) that the thyroxine-albumin interaction is the same in normal and abnormal states. These assumptions obviously require evaluation and clearly limit any reliance on the calculated free thyroxine values in an absolute sense. The most questionable assumptions (*d* and *e*), however, would not affect the calculation when used as a basis for comparison between groups of subjects.

A further consideration with respect to the calculations in pregnancy is the possibility that the increment in serum PBI may not be thyroxine. The solubility of this PBI in butanol (6) is consistent with it being thyroxine, but is inconclusive. One chromatographic study of postpartum serum (4), however, could demonstrate only thyroxine and triiodothyronine.

As indicated in the Methods section, precise knowledge of the concentration of albumin is not critical to the calculations in the present study. For example, in a normal serum with 10^{-7} M thyroxine and 2.7 times 10^{-7} M TBP sites for thyroxine, a 50 per cent change in albumin concentration from the value employed results in approximately a 10 per cent change in free thyroxine in the opposite direction.[3] In a pregnancy serum with 1.4 times 10^{-7} M thyroxine and 5.1 times 10^{-7} M TBP sites, a 50 per cent change in albumin results in a 2 per cent change in free thyroxine. For this reason, albumin was not measured in the individual sera, and the gradual fall in serum albumin which occurs during pregnancy was disregarded. This simplification, of course, could not be applied to all situations. In nephrosis, for example, when the concentration of both albumin and TBP may be very low (10), the albumin level becomes of considerable importance in determining the concentration of free thyroxine.

The findings in the newborn subjects are of interest with regard to the question of the dependence of the fetus on maternal thyroid function. The existence of an independent fetal thyroid function in the human is supported by the ability of hypothyroid women to give birth to apparently normal infants (32) and, conversely, by the finding of retarded bone age in newborn athyreotic infants of normal mothers (33). The slow passage of maternal thyroxine into the fetus (34) indicates that TBP does not readily pass the placental barrier, if at all, and that the placenta probably does not transfer free thyroxine by any mechanism other than diffusion. It appears, therefore, that the higher concentration of free thyroxine in the fetal circulation must be maintained by the fetal thyroid gland. The demonstration of a difference between the concentration of TBP in maternal and fetal blood, which corroborates the findings of Dowling, Freinkel, and Ingbar (13), is not surprising in view of differences in the concentration of other serum proteins (22, 35), and it is of interest that the alpha-1 and alpha-2 globulin components are lower in fetal blood.

The two infants studied on the third day after birth did not appear to have PBI levels higher than that in placental blood, as has been described in infants of this approximate age (36, 37). Measurement of TBP in a larger group of in-

[3] The change in free thyroxine is greater when albumin decreases than when albumin rises (11 per cent and 9 per cent, respectively).

fants and young children will be required to determine whether TBP changes may persist independent of the influence of the high maternal estrogen level.

Since pregnancy and estrogen administration produce increases in PBI and TBP in the serum, it appears possible that similar alterations might occur in women during the menstrual cycle. Attempts to detect such changes in the PBI revealed no unequivocal cyclic variation (5, 38). In the nonpregnant subjects who served as controls for the present study, no significant differences between TBP in men and women were observed (15), but the time of sampling was not correlated with the menstrual cycle. Recent studies on the baboon, however, have revealed a rise in PBI during the postovulatory phase (39). It will be of interest to determine whether this PBI change accompanies a cyclic variation in TBP.

The literature quoted in this discussion, it should be pointed out, has been limited to studies in man, except for that referred to just above. Although considerable data has been recorded on thyroid interrelationships in pregnant animals, differences in placentation and in thyroxine-protein interactions (40) among the species make it hazardous to apply these findings to human pregnancy.

SUMMARY

The thyroxine-binding capacity of the thyroxine-binding alpha globulin of serum (TBP) has been measured during pregnancy in normal women. This capacity, which probably reflects the concentration of TBP, rises in the early weeks of pregnancy to a level which is approximately two and one-half times that in normal adults, remains essentially unchanged throughout pregnancy, and returns to normal within six weeks post partum. As a result of this rise in TBP, the concentration of free thyroxine in serum, calculated with the aid of certain assumptions, is significantly lower than that in nonpregnant adults, despite a rise in serum PBI.

The thyroxine-binding capacity of TBP in the newborn is approximately one and one-half times that in normal adults, and is lower than that in the mother. Since the PBI in the newborn is approximately equal to that in the mother, the concentration of free thyroxine is significantly higher. This level, however, is the same as that in nonpregnant adults.

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REFERENCES

1. Mussey, R. D. The thyroid gland and pregnancy. Amer. J. Obstet. Gyn. 1938, **36**, 529.
2. Freedberg, I. M., Hamolsky, M. W., and Freedberg, A. S. The thyroid gland in pregnancy. New Engl. J. Med. 1957, **256**, 505.
3. Pochin, E. E. The iodine uptake of the human thyroid throughout the menstrual cycle and in pregnancy. Clin. Sci. 1952, **11**, 441.
4. Ferraris, G. M., and Scorta, A. Comportamento di alcuni tests di funzione tiroidea in gravidanza e in puerperio. Minerva ginec. 1955, **7**, 308.
5. Heinemann, M., Johnson, C. E., and Man, E. B. Serum precipitable iodine concentrations during pregnancy. J. clin. Invest. 1948, **27**, 91.
6. Danowski, T. S., Gow, R. C., Mateer, F. M., Everhart, W. C., Johnston, S. Y., and Greenman, J. H. Increases in serum thyroxine during uncomplicated pregnancy. Proc. Soc. exp. Biol. (N. Y.) 1950, **74**, 323.
7. Sandiford, I., and Wheeler, T. The basal metabolism before, during, and after pregnancy. J. biol. Chem. 1924, **62**, 329.
8. Burwell, C. S. Circulatory adjustments to pregnancy. Johns Hopk. Hosp. Bull. 1954, **95**, 115.
9. Gordon, A. H., Gross, J., O'Connor, D., and Pitt-Rivers, R. Nature of the circulating thyroid hormone-plasma protein complex. Nature (Lond.) 1952, **169**, 19.
10. Robbins, J., and Rall, J. E. The interaction of thyroid hormones and proteins in biological fluids *in* Recent Progr. Hormone Res. New York, Academic Press Inc. 1957, vol. 13, p. 161.
11. Robbins, J. Thyroxine-binding capacity of serum in pregnancy. Fed. Proc. 1956, **15**, 153.
12. Dowling, J. T., Freinkel, N., and Ingbar, S. H. Thyroxine-binding by sera of pregnant women. J. clin. Endocrin. 1956, **16**, 280.
13. Dowling, J. T., Freinkel, N., and Ingbar, S. H. Thyroxine-binding by sera of pregnant women, new-born infants, and women with spontaneous abortion. J. clin. Invest. 1956, **35**, 1263.
14. Robbins, J., and Rall, J. E. Thyroxine-binding capacity of serum in normal man. J. clin. Invest. 1955, **34**, 1324.
15. Robbins, J. Reverse-flow zone electrophoresis. A method for determining the thyroxine-binding capacity of serum protein. Arch. Biochem. Biophys. 1956, **63**, 461.

16. Zak, B., Willard, H. H., Myers, G. B., and Boyle, A. J. Chloric acid method for determination of protein-bound iodine. Analyt. Chem. 1952, **24**, 1345.
17. Mack, H. C. The Plasma Proteins in Pregnancy. Springfield, Charles C Thomas, 1955.
18. Robbins, J., Rall, J. E., and Petermann, M. L. Thyroxine-binding by serum and urine proteins in nephrosis. Qualitative aspects. J. clin. Invest. 1957, **36**, 1333.
19. Snedecor, G. W. Statistical Methods, 4th ed. Ames, The Iowa State College Press, 1947.
20. Robbins, J., and Rall, J. E. Effects of triiodothyronine and other thyroxine analogues on thyroxine-binding in human serum. J. clin. Invest. 1955, **34**, 1331.
21. Larson, F. C., and Albright, E. C. The specificity of thyroxine binding by serum alpha globulin. Endocrinology 1955, **56**, 737.
22. Longsworth, L. G., Curtis, R. M., and Pembroke, R. H., Jr. The electrophoretic analysis of maternal and fetal plasmas and sera. J. clin. Invest. 1945, **24**, 46.
23. Coryell, M. N., Beach, E. F., Robinson, A. R., Macy, I. G., and Mack, H. C. Metabolism of women during the reproductive cycle. XVII. Changes in electrophoretic patterns of plasma proteins throughout the cycle and following delivery. J. clin. Invest. 1950, **29**, 1559.
24. Engstrom, W. W., and Markardt, B. Influence of estrogen on thyroid function. J. clin. Endocrin. 1954, **14**, 215.
25. Dowling, J. T., Freinkel, N., and Ingbar, S. H. Effect of diethylstilbestrol on the binding of thyroxine in serum. J. clin. Endocrin. 1956, **16**, 1491.
26. Robbins, J. Unpublished data.
27. Smith, G. VanS., and Smith, O. W. Internal secretions and toxemia of late pregnancy. Physiol. Rev. 1948, **28**, 1.
28. Robbins, J., and Rall, J. E. Physiological implications of thyroxine-binding by serum proteins (abstract). J. clin. Invest. 1957, **36**, 923.
29. Danowski, T. S., Schwartz, R., Rhodes, G. J., Yoder, R. D., Puntereri, A. J., and Mateer, F. M. Serum protein-bound iodine levels following administration of thyroxine in various diseases. J. clin. Endocrin. 1955, **15**, 1116.
30. Astwood, E. B. The use of antithyroid drugs during pregnancy. J. clin. Endocrin. 1951, **11**, 1045.
31. Hamolsky, M. W., Stein, M., and Freedberg, A. S. The thyroid hormone-plasma protein complex in man. II. A new *in vitro* method for study of "uptake" of labelled hormonal components by human erythrocytes. J. clin. Endocrin. 1957, **17**, 33.
32. Parkin, G., and Greene, J. A. Pregnancy occurring in cretinism, and in juvenile and adult myxedema. J. clin. Endocrin. 1943, **3**, 466.
33. Dorff, G. B. Sporadic cretinism in one of twins. Amer. J. Dis. Child. 1934, **48**, 1316.
34. Grumbach, M. M., and Werner, S. C. Transfer of thyroid hormones across the human placenta at term. J. clin. Endocrin. 1956, **16**, 1392.
35. Moore, D. H., Du Pan, R. M., and Buxton, C. L. An electrophoretic study of maternal, fetal, and infant sera. Amer. J. Obstet. Gynec. 1949, **57**, 312.
36. Danowski, T. S., Johnston, S. Y., Price, W. C., McKelvey, M., Stevenson, S. S., and McCluskey, E. R. Protein-bound iodine in infants from birth to one year of age. Pediatrics 1951, **7**, 240.
37. Man, E. B., Pickering, D. E., Walker, J., and Cooke, R. E. Butanol-extractable iodine in the serum of infants. Pediatrics 1952, **9**, 32.
38. Danowski, T. S., Hedenburg, S., and Greenman, J. H. The constancy of the serum precipitable or protein-bound iodine in healthy adults. J. clin. Endocrin. 1949, **9**, 768.
39. Van Zyl, A. Serum protein-bound iodine and serum lipid changes in the baboon (Papio ursinus). I. During the menstrual cycle. J. Endocrin. 1957, **14**, 309.
40. Robbins, J., Apter, R. A., and Rall, J. E. Thyroxine-protein interactions in the serum of various animal species. Program of the Endocrine Society, Thirty-ninth Meeting, 1957, p. 50.

CAROTENOIDS IN MAN. III. THE MICROSCOPIC PATTERN OF FLUORESCENCE IN ATHEROMAS, AND ITS RELATION TO THEIR GROWTH [1]

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In a previous study of the carotenoid content of human aortic atherosclerosis it was concluded by Blankenhorn, Freiman, and Knowles that these dietary lipids accumulate in direct proportion to the extent and relative age of the lesions in which they occur. A series of graded aortic segments was assayed, and it was found that increased severity of atherosclerosis was accompanied by a parallel increase in the concentration of carotenoids (1). Exact location of these pigments in relation to the microscopic anatomy of atherosclerotic lesions should aid in evaluation of the role of exogenous lipids of this class in the initiation of atheromas and their continued growth. It seemed possible that this might be accomplished by fluorescence microscopy, since carotenoids and vitamin A, on exposure to ultraviolet light, produce a characteristic pale green fluorescence by which they can be exactly localized and roughly quantitated in tissue sections (2). Although Popper (2), and Hamperl (3), who performed surveys of the fluorescence in human tissue, reported no evidence of carotenoids or vitamin A in atheromas, it was decided to reinvestigate this matter in view of the findings of Blankenhorn, Freiman, and Knowles. The surveys of Popper and Hamperl were performed on tissues fixed in formalin, a procedure known to diminish the fluorescence of carotenoids and vitamin A. Therefore, in the study here reported unfixed atheromas were examined. Green fluorescence was found and evidence will be presented to show that this fluorescence is a valid means of localizing carotenoids and/or vitamin A in atheromatous lesions. As a class, early lesions show little pale green fluorescence, whereas more advanced lesions consistently have this fluorescence distributed through their lipid-bearing areas in an apparently random manner. These findings are in accord with the previously published data from chemical assay (1) and provide further evidence that those lipids which accumulate as atherosclerotic lesions increase in size and relative age include certain dietary lipids.

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MATERIALS AND METHODS

Twenty whole aortas obtained at autopsy were examined after storage for short periods of time at refrigerator temperature. The 42 plaques studied were of Grades II to IV, according to a published grading scheme which assigns a grade of IV to the most advanced lesions of atherosclerosis (1). Unfixed frozen sections 15 microns thick, mounted in water on glass slides, were examined with ultraviolet light, and adjacent serial sections, stained with Oil Red O and hematoxylin and eosin, were studied with visible light. A representative segment of each plaque was also fixed in formalin, embedded in paraffin for sectioning, and stained with hematoxylin and eosin. The most satisfactory procedure evolved to date for localizing the fluorescence found in plaques has been by comparison with Oil Red O stained frozen sections at the time of fluorescence microscopy. When necessary, heavily calcified plaques were immersed for periods up to seven days in 10 per cent ethylenediaminetetracetic acid tetrasodium salt (EDTA) at refrigerator temperatures. This has been avoided whenever it appeared possible to cut adequate sections without decalcification, since following this procedure, tissues have a blue cast by ultraviolet light. Efforts to detect green fluorescence are more difficult under these conditions.

The source of ultraviolet light was a Hanovia No. 16106 Mercury Vapor Lamp with a dark red-purple glass filter, which emits maximum energy between 3600 Å. and 3700 Å. Observations for fluorescence were made with a monocular microscope, screened by a cardboard shield in such a manner as to allow only the substage and mirror to be exposed to ultraviolet light. An alumi-

TABLE I

The four types of fluorescence found in human atheromas

Appearance	Distribution	Source
Pale green, occasionally has a faintly blue cast. Fades in 15 to 30 seconds.	A diffuse, amorphous haze. Occasionally small discrete granules.	Vitamin A Carotenoids
Dim, pale green, or pale pea-green. Occasionally a faintly blue cast. Fades slowly but not completely, or may not fade.	A diffuse, amorphous haze. Occasionally small discrete granules.	Carotenoids
Brilliant yellow or golden brown. Does not fade.	Discrete, irregular, or oblong crystalline masses which often appear in clumps.	Unknown Sterols?
Bright, light blue or bluish-gray. Does not fade.	Discrete, irregular masses or circlets which often appear in clumps. Occasionally a diffuse, amorphous haze.	Unknown Calcium? (the discrete masses)

nized mirror (American Optical No. 363) was fitted to the microscope. A yellow filter, supplied with this equipment for use in the microscope ocular to prevent corneal injury with prolonged observation, was not used after it was found to obscure areas of faint fluorescence. Observations were carried out in such a way as to limit the exposure of one individual to short periods not more than twice a week.

RESULTS

A. The fluorescence of atheromas

With the equipment as described, background illumination is deep purplish-red, and opaque nonfluorescent objects are black. Intact media appears as a brilliant network of sky-blue linear fibers observed against a dark background, suggesting that individual elastic fibers can be visualized against nonfluorescing ground substances. However, blue fluorescence is not specific for elastic tissue since other substances, including lint and cellulose fibers, have this property. Media in areas involved by atheromata does not have this appearance due to loss of elastic fibers. Thus, the extent of medial involvement by atherosclerosis can be readily judged. The effect of pathologic changes other than those of atherosclerosis involving media have not been studied.

In atherosclerotic plaques, fluorescence of four different types is encountered. These types are described in Table I.

Evidence which indicates that the two types of green fluorescence described can be attributed to carotenoids or vitamin A is provided by the work of Popper and Greenberg (4) and von Querner (5). Additional data supporting this view will be presented below. The source of yellow, golden-brown, and blue fluorescence is not known. The bright yellow and gold bodies have birefringence when examined by polarized light, which suggests that they may be crystallized sterols. When oblong, they are the size and shape of the "cholesterol clefts" seen in atheromas, and they have been observed in areas containing such clefts. Golden-brown and yellow bodies have been reported by Popper to occur in a number of tissues and have been attributed to lipofuscins (2), but not to sterols. The blue circlets and granular bodies are often found in areas showing focal calcification.

In 42 plaques (17 Grade II, 12 Grade III, 13 Grade IV) the type of fluorescence encountered has been classified and correlated with the pathologic anatomy of the lesions. Each grade of severity presents a characteristic pattern.

Grade II plaques. Plaques of this class are yellow or yellowish-white and opaque upon gross examination. Microscopic examination of stained sections shows that distinct plaque formation is present, with only minimal overlying fibrosis and little or no medial involvement. By ultraviolet light these plaques show very little fluorescence. The media makes up the bulk of the thickness of the aorta, the plaque appearing as a dark area in which faint fluorescence can be observed with some difficulty. The fluorescence present appears as dim, nonfading streaks of green, or homogenous small patches of green, which occasionally appear to fade but are usually so faint that the occurrence of fading cannot be judged accurately. It is found in thin streaks, distributed at random throughout the lipid-bearing areas of the plaque. Occasionally, it may appear concentrated either at the inti-

mal or medial border of the plaque. Plaques may be encountered which show almost no fluorescence despite the presence of Oil Red O staining material. It should be noted, however, that such lesions may be yellow by visible light and that carotenoids in trace amounts can be extracted from them, and so it seems possible that with more sensitive equipment fluorescence might be visualized in these plaques.

Scattered, small, round, yellow, or golden-brown bodies are not uncommon and some have a green tint. They may be single or appear in clusters of four or five, and they appear most commonly along the medial (external) border of the plaque. Fibrous areas in the plaque show no fluorescence and appear dark, except for an occasional nonfading yellow granule.

Grade III plaques. Plaques of this class are white or gray, with thick overlying fibrous caps covering a mass of atheromatous material contained between the fibrous cap and the media. Usually this mass is streaked with yellow and is a solid or semisolid material which contains occasional calcified particles. Rarely, it is semiliquid. On microscopic examination of stained sections, the intimal cap is usually intact, with little or no ulceration, and may be quite thick. Extracellular lipid makes up a major portion of the plaque, and moderately extensive damage to the media is usually present. By ultraviolet light, green fluorescence of the fading and nonfading types is easily found and is scattered in a random manner through all areas which can be stained with Oil Red O. Single sections rarely show an even distribution of this fluorescence; but when serial sections are examined, a random pattern within lipid-bearing areas is the rule. The intensity of the fluorescence varies from bright to dull, and various shades of green may be seen in the same section. Green fluorescence usually occurs as a diffuse, amorphous haze with clumplike areas of increased intensity which fade at various speeds or may not fade at all. Occasionally it appears as small, discrete, irregular bodies which fade. Scattered through the background of amorphous fluorescence are bright, irregular, cuboid bodies, either golden, yellow, or blue. The golden or yellow bodies may be oblong and resemble cholesterol clefts. Decalcification with EDTA imparts a blue tint and appears to diminish areas of green fluorescence but does not alter the golden, yellow, or blue fluorescent bodies.

Grade IV plaques. These are the most advanced lesions of atherosclerosis. Atheromatous ulcers, necrotic plaques with hemorrhage, and those with large deposits of calcium are included. All show marked medial involvement. Microscopic examination of intact stained sections shows large masses of extracellular lipid, extensive medial damage, and ulceration. With ultraviolet light, these lesions present the most marked fluorescence seen in atherosclerotic plaques. Green fluorescence, bright and dim, fading and nonfading, is present in abundance as an amorphous haze, randomly distributed through the lipid-bearing areas. Cuboid bodies of bright green are seen, often in the neighborhood of clustered, golden, or blue nonfading fluorescent bodies, which are present in greater number than in less advanced lesions. Areas which appear to be in transition from recognizable media to atherosclerotic plaque can be found. Here the medial fibrils are thinner and have lost their definition and brilliance. The normally dark interspaces between fibrils are filled with homogenous green fluorescent material which fades in patches.

Loss of necrotic material in sectioning and the necessity of prolonged decalcification make the examination of Grade IV plaques difficult. EDTA imparts a blue cast to the diffuse background fluorescence, but green, fading fluorescence can be found with ease. Upon occasion, sections were not attempted because calcific plates, semiliquid atheromatous material, and the adventitia were all that remained of the aortic wall. Smears of these lesions revealed green fluorescence in abundance with scattered golden and blue bodies.

B. The identity of green fluorescence in atheromas

Previously, it has been shown that the yellow pigments extracted from normal and atherosclerotic aortas with ethanol-petrol ether are carotenoids and that these carotenoids will be absorbed onto alumina from hexane and eluted with ethanol-hexane (6). Ethanol-petrol ether extracts of atheromas, yellow by visible light, show a green fluorescence identical with that seen in plaques when dried and examined by ultraviolet microscopy. This examination is made more con-

venient by placing a few granules of anhydrous sodium sulfate on the slide. The fluorescence of a local concentration of lipids around these granules is more easily found than that of the thin oily film which results when extracts are dried on clean glass. Sodium sulfate granules are dark by ultraviolet light and do not fluoresce until extracts are dried upon them, whereupon they assume the fluorescence of adjacent or adsorbed lipids. After examination, the fluorescent lipid can be recovered by washing with petrol ether, leaving dark granules.

Two experiments are reported in which the fluorescent substances found in atheromas were extracted, chromatographed on alumina, and found to have the mobility of carotenoids and/or vitamin A.

Experiment 1. Approximately one-tenth of a ml. of semiliquid homogenous yellow atheromatous porridge was scooped from the cut surface of an iliac arterial plaque which had a thick overlying fibrous cap and an intact adventitia. A thick smear of this material was opaque to visible light, and one corner of the smear examined by ultraviolet light showed diffuse bright and dim green fluorescence, both fading and nonfading. The smear was extracted with (10:100) ethanol-petrol ether (30 to 60° C.) by gently flowing this solvent over the slide and collecting the effluent. All green fluorescence was immediately removed from the thin areas of the slide, although these areas remained opaque to visible light and the bulk of the material present could be seen to have remained fixed to the slide. Repeated washes left only a few yellow-green particles in the thickest parts of the slide which could not be washed free of fluorescence. The collected washes, reduced to dryness at 20° C. with water vacuum and taken up in several drops of hexane (Skellysolve B), showed a very faint yellow color. One drop was examined by ultraviolet light on sodium sulfate granules and showed green fluorescence. The remainder was charged onto a 30 by 1 mm. column of alumina prepared in a Pasteur capillary pipette. A sharply defined, strongly adsorbed, pigment band developed which did not move with hexane but immediately passed off the column with ethanol-hexane (8:100). The ethanol-hexane (8:100) fraction was collected and when concentrated with water vacuum showed a faint yellow color by visible light and green fluorescence when examined on granules of sodium sulfate by ultraviolet light.

Experiment 2. A segment of abdominal aorta showing severe atherosclerosis was stripped of adventitia and assayed by a previously published technique (6). One ml. of an ethanol-petrol ether (10:100) extract of this aorta, containing 2.4 μg. total carotenoids (39 per cent Fraction I, 44 per cent Fraction II, 17 per cent Fraction III),[3] 16 milligrams total sterols (Lieberman-Burchard reaction) and 24 milligrams total lipid, was charged onto a 10 by 100 mm. column of alumina and the chromatogram developed with acetone-hexane (20:100) until the pigments of Fractions I, II and III were seen to be separated. The chromatogram was then cut into segments, extracted and examined for fluorescence on sodium sulfate granules. From the top of the chromatogram downward the following zones were present:

Zone 1—A 0.25 cm. sharp, yellow band (Fraction III) showed nonfading green fluorescence.

Zone 2—A 1 cm. diffuse, orange band (Fraction II) showed a dimmer, nonfading metallic green fluorescence.

Zone 3—A 2.5 cm. clear zone showed no fluorescence.

Zone 4—A 2 cm. sharp, orange band which was skewed (Fraction I) showed greenish-blue nonfading fluorescence with patches of bright pea-green fluorescence which faded in 20 to 40 seconds.

[3] If all carotenoids reported to occur in human tissue were present in an extract subjected to this fractionation, the fractions obtained would contain the following:

Fraction I	*Fraction II*	*Fraction III*
Zeta carotene	Prolycopene	Cryptoxanthin
Alpha carotene	Lycopene	Lutein
Beta carotene	An "unknown" carotenoid	Zeaxanthin
	Diesters of carotenols found in Fraction III	Capsanthin
		Capsorubin

Vitamin A alcohol would migrate with Fraction III; esterified vitamin A would migrate with Fraction I.

Zone 5—A 2 cm. colorless zone was nonfluorescent.

Zone 6—A 2 cm. colorless zone was nonfluorescent.

When the material obtained from all fluorescent zones was recombined and dried upon sodium sulfate, it showed fluorescence of the same shade of green as was present in a sample of unseparated extract.

These experiments provide evidence that essentially all of the green fluorescence in atheromas is extracted with ethanol-petrol ether (10:100) and, once extracted, all of this fluorescent material behaves on a column of alumina in a manner known to be characteristic of carotenoids. Since vitamin A migrates with carotenoids on the chromatograms described, these experiments do not serve to differentiate the separate contributions of vitamin A and carotenoids to the fluorescence seen in atheromas. Examination of the ultraviolet spectrum of the extract used in Experiment 2, and that from five other aortas, indicates that only relatively little vitamin A can be present in proportion to the concentrations of carotenoids.

DISCUSSION

It is believed that the chromatographic experiments here reported on the identity of green fluorescence in atheromas, and the previous work of Popper and Greenberg (4) and von Querner (5) on the identity of the green fluorescence in other tissues, make it valid to assume that either carotenoids and/or vitamin A are present in those parts of the atheroma in which green fluorescence is seen. The known functions of carotenoids and vitamin A in man are vastly different (certain carotenoids can serve as vitamin A precursors, the remainder have no known function), and differences exist in their transport by serum (7) and storage by tissue (8). However, both carotenoids (beta carotene, $C_{40}H_{56}$; lutein, $C_{40}H_{56}O_2$) and vitamin A ($C_{20}H_{30}O$) are large lipid molecules which cannot be synthesized by human tissues, and so the distribution of either serves to localize a dietary lipid in the lesions of atherosclerosis. Our findings indicate that dietary lipids of this class are distributed in a random fashion throughout the lipid-bearing areas of atherosclerotic lesions.

Accumulation of carotenoids and/or vitamin A does not appear affected by thick layers of overlying fibrosis nor does it bear any discernible relation to intramural hemorrhage. It is worthy of note that advanced lesions with large lipid masses show more and brighter fluorescence than early, small ones, suggesting that in addition to an increased amount of lipid, late lesions contain an increased concentration of carotenoids and/or vitamin A in this lipid. This aspect of our study supports the current view that although lipids of early atherosclerotic lesions are quite similar to those found in serum, later lesions show a higher proportion of less polar lipids (9, 10), and suggests that selective accumulation of certain serum lipids rather than some other process may be responsible. Certainly, it seems clear that the carotenoid and/or vitamin A content of the lipid in atheromas are not diluted by the further accumulation of lipid as plaques grow, and this finding by fluorescence microscopy supports the result previously obtained by chemical assay (1).

It is possible that distinction between the presence of carotenoids and vitamin A in atheromata may assume more importance than is attached to it in this report. Carotenoids, as such, are considered to be relatively inert in human tissue, but vitamin A and its fluorescent analogues with biologic activity might produce changes in the character of atherosclerotic lesions. Popper and Greenberg have shown that in certain situations differentiation between carotenoids and vitamin A can be made on the basis of intensity of fluorescence and the rate at which it fades (4). However, since the fluorescence of vitamin A may vary with the location in which it is found, its concentration, and the conditions of examination (2), fractionation of the unsaponifiable material from atheromas would seem to be a more reliable means. To date, examination of ultraviolet curves from extracts of six aortas indicates that the vitamin A content must be small in relation to the concentration of carotenoids.

Our findings at present seem applicable only to the continued growth of atheromas and do not shed light on the initiation of plaque formation. It is possible that the factors which initiate atherosclerosis are different from those which cause progression of the lesions. Systematic study of the sequential development of atheromas might suggest some means by which progression may be

retarded. In view of the fact that most of the pathologic effects of atherosclerosis are associated with the occurrence of advanced lesions rather than early ones, this area of investigation would appear fruitful. One aspect of the change from small, early plaques to large, advanced ones is accumulation of lipid through which carotenoids and/or vitamin A have been shown to occur at random. Study of the accumulation of carotenoids and vitamin A should help to clarify mechanisms of lipid deposition in advancing atheromas. Further investigation of the fluorescent pattern of atheromas with more sensitive equipment might reveal information unrecognized at present. In addition, the study of carotenemia or hypervitaminosis A in man and experimental animals should provide a means by which the mechanism of the accumulation of these two exogenous lipids in atheromas may be investigated directly.

CONCLUSIONS

1. The occurrence of green fluorescence, fading and nonfading, in human atheromas examined microscopically by ultraviolet light is reported.

2. This fluorescence is scattered at random through lipid-bearing areas of plaques and is seen in greater amounts in more advanced lesions.

3. Evidence is presented that this fluorescence is a valid means by which carotenoids and/or vitamin A, two lipids of dietary origin, can be localized in the lesions of atherosclerosis.

4. These findings do not appear to apply to present theories regarding the initiation of atheromas. They suggest that increase in size and severity of atherosclerotic lesions is accompanied by a selective accumulation of certain nonpolar lipids, some of which are derived from diet.

ACKNOWLEDGMENT

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REFERENCES

1. Blankenhorn, D. H., Freiman, D. G., and Knowles, H. C., Jr. Carotenoids in man. I. The distribution of epiphasic carotenoids in atherosclerotic lesions. J. clin. Invest. 1956, **35**, 1243.
2. Popper, H. Histologic distribution of vitamin A in human organs under normal and under pathologic conditions. Arch. Path. 1941, **31**, 766.
3. Hamperl, H. Die Fluorescenzmikroskopie menschlicher gewebe. Virchows Arch. 1934, **292**, 1.
4. Popper, H., and Greenberg, R. Visualization of Vitamin A in rat organs by fluorescence microscopy. Arch. Path. 1941, **32**, 11.
5. von Querner, F. R. Der mikroskopische Nachweis von Vitamin A im animalen gewebe. Klin. Wschr. 1935, p. 1213, II Haljahr.
6. Blankenhorn, D. H. Carotenoids in man. II. Fractions obtained from atherosclerotic and normal aortas, serum, and depot fat by separation on alumina. J. biol. Chem. 1957, **227**, 963.
7. Krinsky, N. I., Cornwell, D. G., and Oncley, J. L. Vitamin A, carotenoids and plasma lipoproteins. Fed. Proc. 1956, **15**, 113.
8. Clausen, S. W. The absorption of Vitamin A and its storage in the tissues. Harvey Lect. Series 38. Lancaster, The Science Press Printing Co., 1943. p. 199.
9. Hirsch, E. F. The chemistry of atherosclerosis in man and its pathogenesis as indicated by animal experiments. Trans. Amer. ther. Soc. 1948, **48**, 113.
10. Page, I. H. Atherosclerosis. An introduction. Circulation 1954, **10**, 1.

STUDIES ON THE BIOCHEMICAL DEFECT OF PERNICIOUS ANEMIA. I. *IN VITRO* OBSERVATIONS ON OXYGEN CONSUMPTION, HEME SYNTHESIS AND DEOXYRIBONUCLEIC ACID SYNTHESIS BY PERNICIOUS ANEMIA BONE MARROW [1, 2]

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In microbiological systems, folic acid and vitamin B_{12} appear to be important in the synthesis of purines and pyrimidines, in the synthesis of methyl groups, and possibly in the synthesis of deoxyribose (1–3). Studies at the clinical level, summarized by Mueller and Will (4), indicate that similar biochemical processes may be involved in patients with pernicious anemia (P. A.). Because such studies on patients are difficult to carry out and interpret, precise methods of *in vitro* assessment of the biochemical activity of P. A. marrow cells are needed.

Direct studies on P. A. marrow have been confined, for the most part, to observations of morphologic changes and cell counts in tissue culture (5–12). In attempting to avoid the difficulties inherent in evaluating these changes in tissue culture and in an effort to secure more direct biochemical information, P. A. marrow has been studied in our laboratory by the following techniques: *1*) oxygen consumption, *2*) heme synthesis as measured by the rate of incorporation of C^{14}-glycine into heme, and *3*) deoxyribonucleic acid (DNA) synthesis by measurement of the rate of incorporation of C^{14}-formate into thymine.

With these techniques, which permit measurements of biochemical activity over short periods of time and with small amounts of bone marrow, we have attempted to answer the following questions: *1*) Does vitamin B_{12} have a direct effect on P. A. marrow cells? *2*) Why has the liquid culture-vaccine vial technique failed to demonstrate an effect of vitamin B_{12}? *3*) Is there an inhibitory factor in P. A. serum? *4*) What is the effect of folic acid on P. A. cells?

METHODS

Bone marrow was obtained by aspiration biopsy from patients with classical pernicious anemia in relapse. The marrow was dispersed by passage through wire screens, and aliquots were pipetted into incubation vessels. The suspending media were varied according to the conditions being studied. In making the various dilutions, care was taken to keep the concentration of serum and of radioactive substrate constant in all aliquots of any given experiment. The methods for measuring oxygen consumption, heme synthesis and DNA synthesis have been described (13, 14). In measuring heme synthesis, the concentration of glycine-2-C^{14} was 1.4 μM per ml. of medium, and the specific activity was 0.2 mc. per mM. In measuring DNA synthesis, the concentration of C^{14}-formate was 0.5 μM per ml. of medium, and the specific activity was 2.0 mc. per mM. At the end of the period of incubation, aliquots of carrier rabbit bone marrow were added to the incubation vessels to facilitate the subsequent isolation of thymine. In any one experiment, therefore, the absolute value of the specific activity of the isolated thymine is a function of the amount of carrier added. Crystalline vitamin B_{12}, folic acid and folinic acid were obtained through the courtesy of Lederle laboratories.

RESULTS

Table I illustrates a comparison, with regard to heme synthesis and oxygen consumption, of P. A. bone marrow incubated in P. A. serum, normal serum, and P. A. serum plus 0.1 γ of vitamin B_{12} per ml. As was indicated previously (13), the comparison of different serum samples is difficult because of a wide range of variability among different normal serum samples from hematologically normal donors. This difficulty is apparent in attempting to compare P. A. serum to

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[2] Presented in part before the National Meeting of the American Federation for Clinical Research at Atlantic City, May 5, 1957.

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TABLE I

*Heme synthesis and oxygen consumption by pernicious anemia bone marrow incubated in pernicious anemia serum, normal serum, and pernicious anemia serum plus 0.1 γ of vitamin B_{12} per ml.**

EXPT.	COMPARISON OF PERNICIOUS ANEMIA SERUM TO NORMAL SERUM AS 100%		COMPARISON OF PERNICIOUS ANEMIA SERUM PLUS B_{12} TO PERNICIOUS ANEMIA SERUM ALONE AS 100%	
	HEME SYNTHESIS	OXYGEN CONSUMPTION	HEME SYNTHESIS	OXYGEN CONSUMPTION
1	127	120		
2	112	99		
3	66	93		
4	126	113		
5	195	102		
6	125	108	89	92
7	100	112	109	103
8	95	91	80	100
9	51	94	110	102
10	108	109	111	99
11	112		102	
12	141	100	104	102
MEAN	113	104	101	100
S.D.	36	9	12	4

* Each experiment represents a different P. A. marrow. The time of incubation for heme synthesis was 10 hours with oxygen consumption being measured during the first 3 hours.

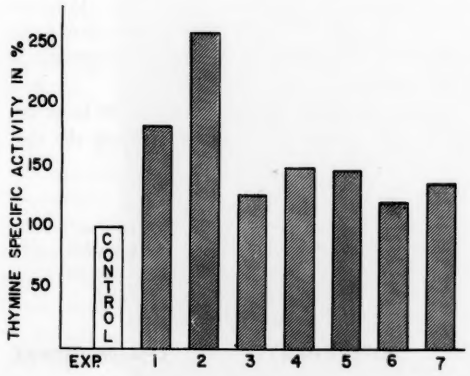

FIG. 1. THE EFFECT OF VITAMIN B_{12} ON DEOXYRIBONUCLEIC ACID SYNTHESIS BY PERNICIOUS ANEMIA MARROW IN PERNICIOUS ANEMIA SERUM

In Experiments 1 and 2, the period of incubation was 10 hours; in Experiments 3 through 7, 5 hours. In Experiments 1 through 5 the concentration of vitamin B_{12} was 0.1 γ per ml; in Experiments 6 and 7, 0.01 γ per ml. In each experiment the control was an aliquot of the P. A. marrow incubated in P. A. serum without added vitamin B_{12}.

normal serum, as indicated by the large standard deviation. In comparing the effect of P. A. serum with and without added vitamin B_{12}, we used aliquots of the same serum in each of the experiments, with the result that the standard deviation is much smaller. It is apparent that, within the limits of the technique, there is no effect of either

TABLE II

The effect of various concentrations of vitamin B_{12} on deoxyribonucleic acid synthesis by pernicious anemia marrow in pernicious anemia serum incubated for five hours

B_{12} γ/ml.	CPM/μM of Thymine
	2830
0.1	3970
0.01	4030
0.001	3920

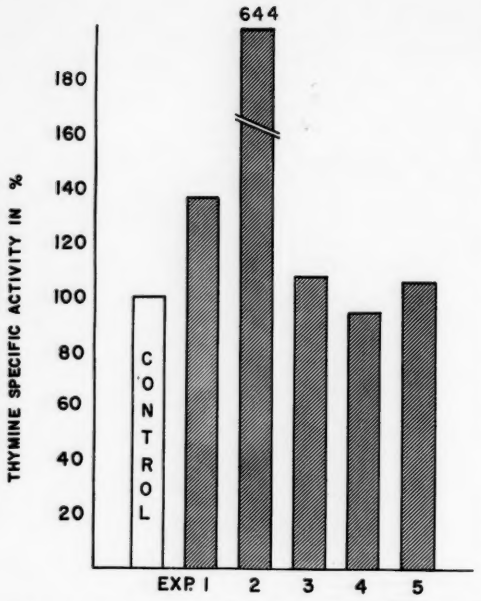

FIG. 2. THE EFFECT OF FOLIC ACID ON DEOXYRIBONUCLEIC ACID SYNTHESIS BY PERNICIOUS ANEMIA MARROW IN PERNICIOUS ANEMIA SERUM

In Experiments 1 and 2 the period of incubation was 10 hours; in Experiments 3 through 5, 5 hours. The concentration of folic acid was 0.2 γ per ml. In each experiment the control was an aliquot of the P. A. marrow incubated in P. A. serum without added folic acid.

normal serum or vitamin B_{12} on these two processes.

Figure 1 illustrates the effect of vitamin B_{12} on DNA synthesis by P. A. marrow cells in P. A. serum. The results illustrated in this figure show that vitamin B_{12} does have a direct and consistent effect at the cellular level. The amount of vitamin B_{12} used in these experiments is considerably above the normal range of 100 to 900 $\mu\mu$g. per ml. (15). The data in Table II show that 0.001 μg. per ml. also produces an optimal effect. In three experiments the addition of vitamin B_{12} to normal serum produced no significant increase in the rate of DNA synthesis by P. A. marrow.

Figure 2 illustrates the effect of folic acid on DNA synthesis by P. A. marrow cells in P. A. serum. In three experiments there was no effect, in one there was a moderate stimulation, and in one there was marked stimulation. In one other experiment, the addition of folic acid to P. A. marrow in normal serum also produced a marked increase in DNA synthesis. It appears, therefore, that P. A. marrow cells differ considerably in their response to folic acid. In three experiments, no difference between the effect of folic acid and of folinic acid was observed.

Table III illustrates two experiments that have been done to test the effect of vitamin B_{12} and folic acid singly and in combination. In Experiment A the marrow was stimulated markedly by folic acid, whereas in Experiment B the folic acid had a barely detectable effect. The effect of the combination of vitamin B_{12} with folic acid was roughly the sum of the individual effects alone. One interpretation of these limited data is that the two substances are not related in their mode of action.

The experiments illustrated in Figures 3A, 3B and 3C were designed to determine whether or not there is an inhibitor of DNA synthesis in P. A. serum. Figure 3A compares the effect of dilution of P. A. serum and normal serum on DNA synthesis by normal marrow, and Figure 3B illustrates a similar experiment using P. A. marrow. DNA synthesis by P. A. marrow and normal marrow in various mixtures of P. A. serum and normal serum is illustrated in Figure 3C. It is apparent that none of these experiments shows any evidence of an inhibitor of DNA synthesis in P. A. serum.

Studies of DNA synthesis over a 48 hour period are illustrated in Figure 4. Using the vac-

TABLE III

The effect of vitamin B_{12} (0.1 γ per ml.), folic acid (0.2 γ per ml.), and both vitamins on deoxyribonucleic acid synthesis by pernicous anemia marrow in pernicious anemia serum incubated for 10 hours

EXPT.	SUPPLEMENT	CPM/μM THYMINE
A	NONE	265
	B_{12}	713
	FOLIC ACID	1,705
	B_{12} & FOLIC ACID	2,410
B	NONE	8,900
	B_{12}	11,300
	FOLIC ACID	9,500
	B_{12} & FOLIC ACID	12,690

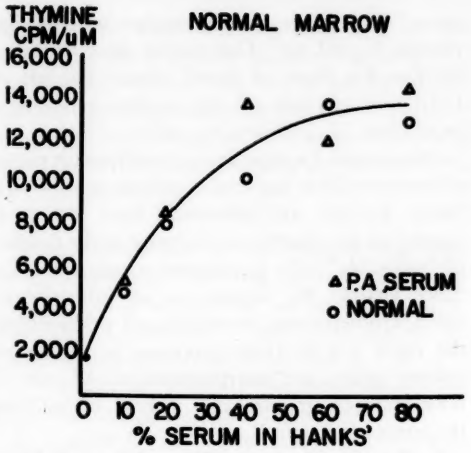

FIG. 3A. THE EFFECT OF DILUTION OF PERNICIOUS ANEMIA SERUM AND NORMAL SERUM ON DEOXYRIBONUCLEIC ACID SYNTHESIS BY NORMAL MARROW

Incubation time was five hours.

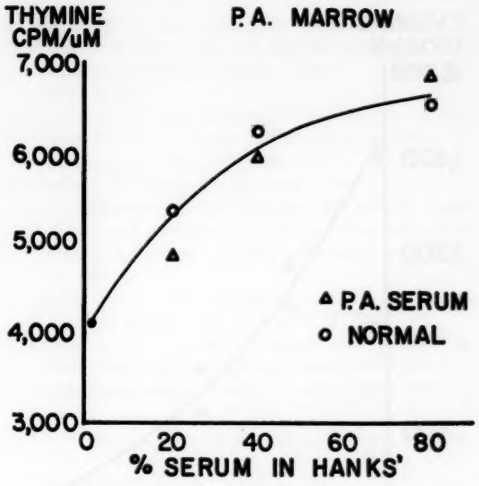

FIG. 3B. THE EFFECT OF DILUTION OF PERNICIOUS ANEMIA SERUM AND NORMAL SERUM ON DEOXYRIBONUCLEIC ACID SYNTHESIS BY PERNICIOUS ANEMIA MARROW

Incubation time was five hours.

cine vial method of Osgood and Brownlee (5), as modified by Lajtha (8), we incubated aliquots of normal marrow in 25 per cent normal serum in Gey's solution. After the various times of incubation, C^{14}-sodium formate was added, and incubation was carried on for an additional four hours. It is apparent from Figure 4 that the ability of marrow cells to synthesize DNA is markedly reduced after 24 hours and has virtually ceased at 48 hours.

DISCUSSION

These short term experiments with P. A. marrow show a definite effect of vitamin B_{12} on DNA synthesis. No effect was demonstrated on either oxygen consumption or heme synthesis. It is possible that longer experiments would show some effect on heme synthesis, as suggested by the experiments of Walsh, Thomas, Chow, Fluharty, and Finch (16). In the first few hours, vitamin B_{12} may permit proliferation of cells and then later, as these cells mature, heme synthesis may begin to increase.

Results given here are of particular interest in that they show a definite action of vitamin B_{12} on P. A. marrow cells. Using the liquid suspension culture technique, Lajtha (7) and Thompson (10) observed that folic acid, but not vitamin B_{12}, had a maturing effect on megaloblasts. These observations led to the speculation that vitamin B_{12} might combine with an intrinsic factor or be altered in some way to form a hematopoietic factor in serum (10). However, the experimental data reported here demonstrate that vitamin B_{12} in P. A. serum has a direct effect on P. A. marrow cells, indicating that an intrinsic factor is not essential to

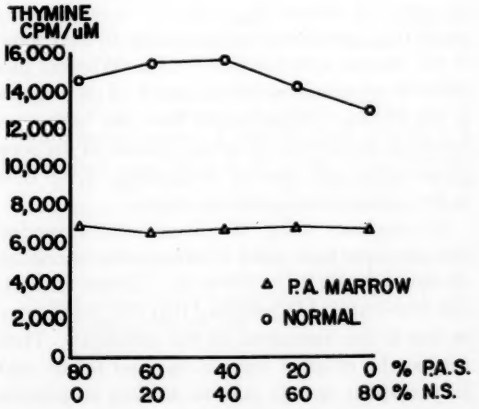

FIG. 3C. THE EFFECT OF VARIOUS MIXTURES OF NORMAL SERUM (N. S.) AND PERNICIOUS ANEMIA SERUM (P. A. S.) ON DEOXYRIBONUCLEIC ACID SYNTHESIS BY PERNICIOUS ANEMIA MARROW AND NORMAL MARROW

Incubation time was five hours.

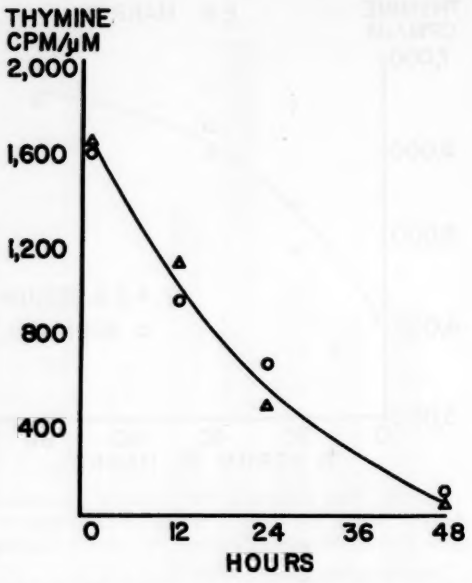

FIG. 4. THE EFFECT OF THE TIME OF PREINCUBATION ON DEOXYRIBONUCLEIC ACID SYNTHESIS BY NORMAL BONE MARROW IN VACCINE VIAL CULTURE

After the period of culture shown, C^{14}-formate was added to the vial. Incubation was then carried on for an additional four hours. In the experiment shown, the triangles represent a nucleated cell concentration of 3,338 per cu. mm. and the open circles represent a concentration of 1,730 per cu. mm.

the action of vitamin B_{12}. The P. A. serum present during incubation has the ability to bind some of the vitamin added *in vitro* (15). Whether this action is important in the utilization of the vitamin is not known. Experiments have not been conducted in the absence of serum because of the poor preservation and lowered metabolism of the cells in the culture media without serum.

Investigators using the liquid culture-vaccine vial technique have failed to demonstrate a cellular effect of vitamin B_{12}. The work of Swan, Reisner, and Silverman (12) indicated that this failure may be due to the limitations of the technique. They studied the effect of vitamin B_{12} and folinic acid on pernicious anemia marrow in both suspension and solid cultures. Vitamin B_{12} appeared to stimulate cell growth in cultures grown on clots and to convert megaloblastic hematopoiesis to normoblastic in cultures grown on glass. In suspension cultures, on the other hand, folinic acid caused an increase in the number of cells, but vitamin B_{12} did not. Our studies show that after the first few hours of liquid culture, the rate of DNA synthesis falls off very rapidly, indicating a rapid loss of proliferative ability (Figure 4). Lajtha observed a rapid loss of proliferative power of marrow cells in suspension cultures *in vitro* (8). Swan, Reisner, and Silverman have also commented on the progressive decrease in the number of "mitotable" cells in suspension cultures (12). Since vitamin B_{12} appears to act primarily on DNA synthesis and associated cell proliferation, the rapid loss of these processes in suspension culture makes the interpretation of 48 and 72 hour cultures an unreliable method of studying the action of vitamin B_{12}.

Lajtha (7), Thompson (9), and Bussi, Erdani, Pozza, Fava, and DeMicheli (11) presented data indicating that P. A. serum possesses the property of inhibiting morphological maturation of both P. A. marrow cells and normal marrow cells. Our technique, however, indicates that P. A. serum does not contain an inhibitor of DNA synthesis. The assessment of maturation by morphologic means is dependent primarily on nuclear changes which may not necessarily be associated with the formation of new DNA. Our results do not permit the exclusion of an inhibitor of morphologic maturation in P. A. serum. However, the above-mentioned limitations of the vaccine vial culture technique cast doubt on the existence of a true inhibitor.

One surprising result has been the marked variation in the response of the marrow to added folic acid. In those P. A. marrow cells responding to folic acid, the magnitude of the effect has been much greater than that of vitamin B_{12}. Nieweg, Faber, de Vries, and Kroese (17) measured the folic acid activity of the whole blood in 16 patients with pernicious anemia. Normal values were found in about half the cases, with the remainder being low. In our experiments, the marrows responding to folic acid may have come from patients who were deficient in folic acid as well as vitamin B_{12}. Blood folic acid levels were not measured.

The technique presented here makes it possible to carry out metabolic studies on marrow cells of man. Experiments along these lines, with various radioactive precursors and with various supple-

mented media, may eventually shed light on the mode of action of vitamin B_{12} and folic acid. It is hoped that some of these studies can be carried out, although the decreasing number of patients with untreated pernicious anemia constitutes a major problem to those working in this field.

SUMMARY

Pernicious anemia (P. A.) marrow has been studied *in vitro* by measuring the rates of oxygen consumption, heme synthesis and deoxyribonucleic acid (DNA) synthesis. From these studies the following conclusions have been drawn:

1. Vitamin B_{12} affects DNA synthesis but not oxygen consumption or heme synthesis.

2. Vitamin B_{12} has a direct effect on DNA synthesis by P. A. marrow cells.

3. Folic acid affects DNA synthesis in different P. A. marrows to a highly variable degree.

4. The liquid suspension-vaccine vial technique is not a suitable method for studies of DNA synthesis by marrow.

5. P. A. serum does not contain an inhibitor of DNA synthesis.

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REFERENCES

1. Ford, J. E., and Hutner, S. H. Role of vitamin B_{12} in the metabolism of microorganisms *in* Vitamins and Hormones. New York, Academic Press Inc. 1955, vol. **13**, pp. 101–136.
2. Pfiffner, J. J., and Bird, O. D. Water-soluble vitamins. I. Vitamin B_{12}, folic acid, choline, and para-aminobenzoic acid. Ann. Rev. Biochem. 1956, **25**, 397.
3. Downing, M., and Schweigert, B. S. Role of vitamin B_{12} in nucleic acid metabolism. IV. Metabolism of C^{14}-labeled thymidine by Lactobacillus Leichmannii. J. biol. Chem. 1956, **220**, 521.
4. Mueller, J. F., and Will, J. J. Interrelationship of folic acid, vitamin B_{12} and ascorbic acid in patients with megaloblastic anemia. Amer. J. clin. Nutr. 1955, **3**, 30.
5. Osgood, E. E., and Brownlee, I. E. Culture of human marrow. Details of a simple method. J. Amer. med. Ass. 1937, **108**, 1793.
6. Astaldi, G., Baldini, M., and Frugoni, C., Jr. Transformazione normoblastica del midolio osseo dell'anemia perniciosa ottenuta in vitro per azione diretta dell'estratto epatico. Haematologica 1948, **31**, 265.
7. Lajtha, L. G. An inhibitory factor in pernicious anaemia serum. Clin. Sci. 1950, **9**, 287.
8. Lajtha, L. G. Culture of human bone marrow *in vitro;* reversibility between normoblastic and megaloblastic series of cells. J. clin. Path. 1952, **5**, 67.
9. Thompson, R. B. Addisonian pernicious anaemia. Confirmatory evidence of a factor inhibiting erythropoiesis. Clin. Sci. 1950, **9**, 281.
10. Thompson, R. B. Observations on the effects of vitamin B_{12}, liver extracts, folic acid and thymine on the maturation of megaloblasts in culture. Blood 1952, **7**, 522.
11. Bussi, L., Eridani, S., Pozza, G., Fava, P. L., and DeMicheli, E. Studi sulla megaloblastogenesi "in vitro" di midolli anemico-perniciosi e normali. Nota I. Biol. lat. (Milano) 1953, **6**, 17.
12. Swan, H. T., Reisner, E. H., Jr., and Silverman, M. The effect of various metabolites on the growth of marrow cells in vitro. Blood 1955, **10**, 735.
13. Thomas, E. D. In vitro studies of erythropoiesis. I. The effect of normal serum on heme synthesis and oxygen consumption by bone marrow. Blood 1955, **10**, 600.
14. Thomas, E. D., and Lochte, H. L., Jr. Deoxyribonucleic acid synthesis by bone marrow cells in vitro. Blood 1957, **12**, 1086.
15. Lear, A. A., Harris, J. W., Castle, W. B., and Fleming, E. M. The serum vitamin B_{12} concentration in pernicious anemia. J. Lab. clin. Med. 1954, **44**, 715.
16. Walsh, R. J., Thomas, E. D., Chow, S. K., Fluharty, R. G., and Finch, C. A. Iron metabolism. Heme synthesis *in vitro* by immature erythrocytes. Science 1949, **110**, 396.
17. Nieweg, H. O., Faber, J. G., de Vries, J. A., and Kroese, W. F. S. The relationship of vitamin B_{12} and folic acid in megaloblastic anemias. J. lab. clin. Med. 1954, **44**, 118.

STUDIES ON THE METABOLISM OF PLASMA PROTEINS IN THE NEPHROTIC SYNDROME. II. THE LIPOPROTEINS[1, 2]

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It is now generally agreed that essentially all of the cholesterol and phospholipid in human plasma is associated with polypeptides in lipid-peptide complexes classified as lipoproteins (1). It is also generally accepted, with some reservations (2), that these lipoproteins can be divided electrophoretically into two major groups (1), α-lipoproteins and β-lipoproteins, which differ from each other with regard to solubility, molecular weight, and content of protein, cholesterol, phospholipid, and neutral fat. The lipoproteins can be differentiated still further according to the density of the individual molecules (3–6), lipoproteins of different densities differing in their relative content of protein and lipid. The relationship of chylomicra to lipoproteins is not yet clear and, although less than 1 per cent of the weight of chylomicra is peptide, chylomicra are frequently, but not universally, considered as lipoproteins.

The lipids in lipoproteins appear to be exchangeable *in vivo* and *in vitro:* cholesterol and phospholipids on α-lipoproteins apparently can exchange with those of β-lipoproteins and *vice versa* (7, 8). The interrelationships of the peptide moieties of lipoproteins, however, are not known. It has been suggested (9) that the peptide moiety of α-lipoprotein goes through a continuous cycle, acquiring lipids to form chylomicra and possibly low density β-lipoproteins; chylomicra and low density β-lipoproteins then release glycerol, fatty acids, and possibly other lipids through the action of lipoprotein lipase to reform α-lipoprotein.

In the present investigation, attention was directed to the metabolism of human serum lipoproteins in an effort to determine the factors responsible for hyperlipemia and hypercholesterolemia in children with the nephrotic syndrome. For this purpose, specific lipoproteins were isolated by ultracentrifugation from normal sera and from sera of children with this disease. The peptide moieties of the isolated lipoproteins were labeled using I^{131} and the turnover and interconversion of these proteins in normal individuals and in children with the nephrotic syndrome were studied.

METHODS

Patients. Six children with the nephrotic syndrome were selected for study (Table I). Three of these had been studied a year previously with regard to their metabolism of albumin and γ-globulin (10); in the interim, one child, K. S., had almost completely recovered from the disease, but the clinical status of the other two children, E. T. and D. W., had remained essentially unchanged. As in the earlier study, the six patients represented three recognizable stages of the disease: Group I, one child in the latent phase of the nephrotic syndrome who had almost complete remission and no longer had hypoproteinemia, ascites, edema, or hyperlipemia, but still had residual proteinuria; Group II, one child with only minimal *intermittent* edema and without ascites but with proteinuria, hypoproteinemia and hyperlipemia; Group III, four children with full-blown disease manifesting proteinuria, hypoproteinemia, anasarca, ascites, and hyperlipemia. Thus, all the children of Groups II and III had hyperlipemia and hypercholesterolemia.

Normal subjects. Eight men and one woman, all between the ages of 21 and 29 years (Table I), were selected as subjects to study the normal metabolism of human plasma lipoproteins. These individuals had normal serum cholesterol levels and showed no evidence of cardiac, renal, or hepatic disease. No attempt was made to regulate their diets during the course of this study

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and there was no restriction of activity. It will be noted that this group was considerably older than the nephrotic children studied. The behavior of the labeled lipoproteins in these adults, however, was similar to that observed in the child, K. S., who had recovered from the nephrotic syndrome.

Isolation of lipoproteins used for iodination. The lipoproteins used in this study were obtained from the serum of individual healthy adults and from the serum of one child, D. O., in Group III. Three major groups of lipoproteins were isolated and iodinated: *1*) low density β-lipoproteins or lipoproteins of density less than 1.000 Gm. per ml.; *2*) higher density β-lipoproteins or those having an average density of 1.038 Gm. per ml.; and *3*) α-lipoproteins of density greater than 1.125 Gm. per ml.

Separation of the β-lipoproteins from normal serum was accomplished by precipitation with dextran sulfate (6). Subsequent dissociation of the β-lipoprotein-dextran sulfate complex was performed by ultracentrifugation (Spinco, Model L, Rotor No. 40) in a sodium chloride density gradient tube at 105,000 times G for 18 hours. In this method, the low density β-lipoproteins floated to the top of the gradient tube and the higher density β-lipoproteins formed a narrow band in the middle portion of the tube; other proteins and the dextran sulfate sedimented to the bottom of the tube. The lipoprotein layers were removed from the gradient tube with the aid of a tube-cutter (11) and aliquots analyzed with an air-driven ultracentrifuge in a sodium chloride solution of density 1.063 Gm. per ml. at 25° C. The Svedberg unit, as modified by de Lalla and Gofman (12), was used to define flotation rates and is signified by the conventional symbol, S_f. The β-lipoprotein patterns were analyzed with the aid of the distribution function developed by Williams, Saunders, and Cicirelli (13) to describe heterogeneous high polymer systems. The analyses revealed that the low density β-lipoproteins prepared by dextran sulfate precipitation contained lipoproteins in the S_f 10 to 100 range with a preponderance of lipoproteins in the S_f 20 to 60 range. The higher density β-lipoproteins were found to be in the S_f 3 to 9 range. Assay for dextran sulfate, based on the effect of dextran sulfate on the prothrombin time of a one-step clotting system, revealed less than 0.5 μg. of dextran sulfate per ml. of lipoprotein fraction (6).

Low density β-lipoproteins were prepared from the serum of Patient D. O. in Group III by direct ultracentrifugal flotation of the serum in a sodium chloride density gradient tube. To prevent contamination of the low density lipoproteins with chylomicra, the serum was first centrifuged at 25,000 times G for 30 minutes; the chylomicra floated to the top of the tube and were separated from the subnatant serum. Analysis of the low-density lipoproteins showed an S_f range of 10 to 200.

The preparation of α-lipoproteins used in this study was kindly supplied by Dr. Ella M. Russ, Cornell Medical School. This preparation was obtained from the plasma of a single donor by low temperature, ethanol-water fractionation (14) and subsequently floated at 100,000 times G in a solution of potassium bromide (15). The solution of α-lipoproteins was dialyzed against 2 M NaCl to remove the KBr before the iodination procedure.

Radioiodinated lipoproteins. The following lipoprotein preparations were iodinated: *1*) low density β-lipoproteins from normal sera, *2*) high density β-lipoproteins from normal sera, *3*) low density β-lipoproteins from nephrotic serum, and *4*) α-lipoproteins from normal serum. I^{131} was obtained as carrier-free NaI in a sodium bisulfite solution and to this was added appropriate amounts of a solution of 0.94×10^{-2} M I_2 in 1.33×10^{-3} M KI (16). The iodide and bisufite were oxidized with nitrous acid, which was subsequently neutralized with sodium hydroxide (17). The mixture was then added to a given lipoprotein solution in 0.2 M $NaHCO_3$-Na_2CO_3 buffer at pH 9.5. The β-lipoproteins were assumed to have a molecular weight of about 2,000,000 and the α-lipoproteins were assumed to have a molecular weight of about 200,000 (1). The iodinated lipoproteins were dialyzed for 24 hours against several changes of 0.15 M NaCl containing 0.05 Gm. per cent of the disodium salt of ethylenediaminetetraacetic acid (EDTA). Immediately after dialysis, sufficient amounts of purified human serum albumin were added to the iodinated lipoproteins to give a final albumin concentration of about 1 Gm. per cent. The preparations were than sterilized by pressure filtration through a sintered glass filter.

Human serum lipoproteins are relatively unstable at room temperatures and are readily oxidized when in the purified state. Consequently, certain precautions were considered essential: *1*) all solutions and sera were kept between 1° C. and 4° C. at all times; *2*) iodination of the lipoprotein fraction was performed within 24 to 48 hours after obtaining blood from the donor; *3*) the radioiodinated lipoproteins were given to the recipients within 24 to 48 hours after iodination; *4*) EDTA, to inhibit oxidation (18), was present in the lipoprotein preparations at all times after iodination and in several instances before iodination as well; and *5*) human serum albumin was added after dialysis and before sterilization to inhibit oxidation and radiation effects (19).

Precipitation of the radioiodinated lipoproteins with an equal volume of 20 per cent trichloracetic acid, in the presence of unlabeled carrier, indicated that less than 2 per cent of the total radioactivity of each preparation was free or loosely bound to the protein. Extraction of the trichloracetic acid precipitates of the iodolipoproteins with organic solvents such as petroleum ether, diethyl ether, ethyl alcohol, and acetone, either singly or in combinations, failed to remove more than 0.5 per cent of the total radioactivity of the precipitates. Denaturation of the iodolipoproteins in the presence of carrier protein by heat and subsequent extraction with organic solvents also failed to remove significant amounts of radioactivity. It appeared that essentially all of the radioactivity in the iodolipoproteins was associated with the peptide moieties of the lipoproteins. It was calculated that the iodinated α-lipoproteins had an average of two atoms of iodine per molecule, the high density β-lipoproteins about one atom of iodine per molecule and the low density β-lipoproteins an average of about 0.8 atom of iodine per molecule.

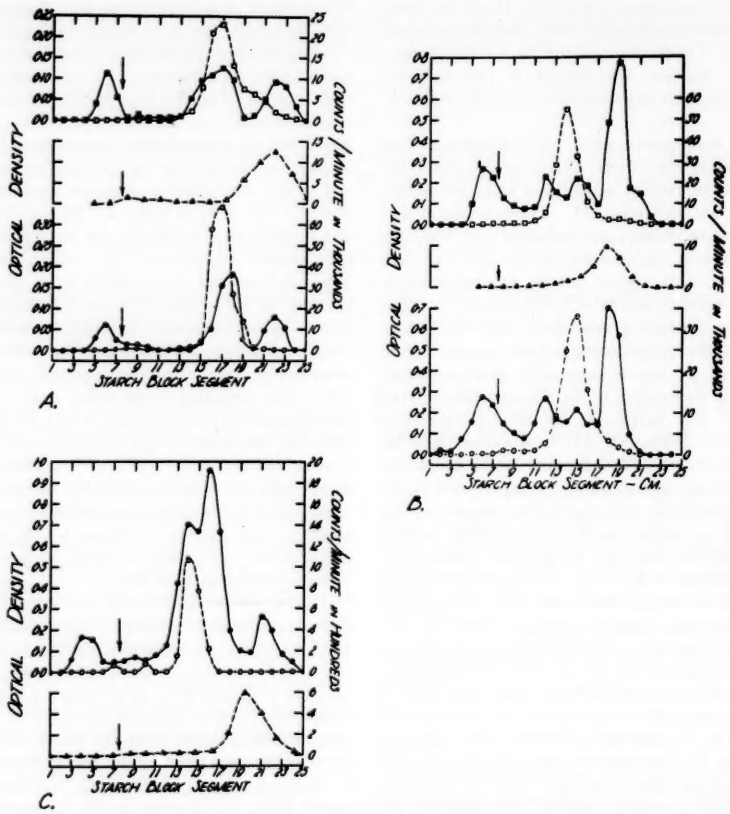

FIG. 1. STARCH ELECTROPHORESIS OF S_f 3 TO 9 LIPOPROTEINS (SQUARES), LOW DENSITY LIPOPROTEINS (CIRCLES) AND α-LIPOPROTEINS (TRIANGLES)

Dashed lines with open symbols represent radioactivity and solid lines with solid symbols represent optical density. Arrows indicate the origin. Starch cut into 1 cm. segments. A. Iodinated lipoproteins in a mixture of unlabeled S_f 3 to 9 lipoprotein, γ-globulin, and albumin. B. Iodinated lipoproteins in plasma from normal individuals after 24 hours *in vivo*. C. Iodinated lipoproteins in plasma from nephrotic children after 24 hours *in vivo*.

There was some evidence that, as the density of the β-lipoproteins decreased, the amount of iodine bound per mol of protein under identical conditions decreased. It was assumed in this study, however, that the degree of labeling within a given lipoprotein fraction was uniform.

When added separately to unlabeled sera or other lipoprotein fractions *in vitro*, 90 per cent or more of the radioactivity of the iodinated lipoproteins was recoverable with the analogous ultracentrifugal fractions.

Zonal electrophoresis in starch (20) was used to study the relative mobility of the native lipoproteins and the radioiodinated derivatives. The starch blocks measured 4 by 30 cm. and were enclosed in waxed paper; electrophoresis was performed in barbiturate-citrate buffer, pH 8.6, $\Gamma/2$ equals 0.1, at 15 volts per cm. for 18 hours at 4° C. The starch was then cut into segments and the proteins eluted with 0.15 M NaCl. The optical densities of the eluates were measured in a Beckman spectrophotometer at a wave length of 280 mμ: the eluates were also assayed for radioactivity. Electrophoretic patterns obtained for the three classes of lipoproteins studied are shown in Figure 1. The mobility of each specific iodolipoprotein was the same when examined either in a mixture of proteins prepared *in vitro* (Figure 1 A) or in serum obtained after their intravenous injection (Figure 1, B and C). It was found that the radioactivity associated with lipoproteins of S_f 3 to 9 or S_f 10 to 200 did *not* shift to the more rapidly migrating α-lipoproteins or *vice versa*, at any time *in vitro* or *in vivo*. The migration of the S_f 3 to 9 lipoproteins was like that of the low

density lipoproteins and, *in this system,* migrated with a mobility between those of the α_2- and β-globulins.

Method of study. The iodolipoproteins were given intravenously. The specific activities of the proteins were such that no more than 1 mg. of any iodolipoprotein was injected per individual. The maximum radioactivity per injection was 0.4 μc. per Kg. body weight. All persons studied were given 10 drops of Lugol's solution three times a day beginning about 24 hours prior to the injection of radioiodolipoprotein.

After the injection of radioiodolipoproteins, samples of venous blood were collected periodically and permitted to clot; the sera were separated and assayed for radioactivity. In this study, serum concentrations and plasma concentrations of lipoproteins were considered equivalent. In many instances, the lipoproteins were separated into the three density classes by ultracentrifugation. The volumes of the separate fractions were measured and the fractions assayed for radioactivity. The amount of cholesterol in each different density class was determined by the method of Abell, Levy, Brodie, and Kendall (21). All sera were centrifuged at 25,000 times G for 30 minutes and separated from the layer of chylomicra at the top *prior* to ultracentrifugal fractionation. S_f analysis of individual fractions of the β-lipoproteins was performed with an air-driven ultracentrifuge.

In several of the patients of Group III, it was possible to obtain large samples of edema and ascitic fluids at the end of the study; these were concentrated fifty- to one hundred-fold by negative pressure dialysis and this fractionated ultracentrifugally.

Urine collections were obtained in five of the normal subjects and in all of the nephrotic patients, the collection periods varying from several hours to several days. Aliquots were assayed for radioactivity.

Nonprotein radioiodide in serum, urine, edema, and ascitic fluid was estimated by adding an equal volume of 20 per cent trichloracetic acid to aliquots, allowing the mixtures to stand at 1° C. for 2 to 24 hours, centrifuging and measuring the radioactivity of the supernatant.

Assay of radioactivity. All samples were counted in a well-type sodium iodide crystal scintillation counter which gave approximately 1×10^6 counts per minute per μc. of I^{131}. Problems of decay and geometry in counting samples were avoided by preparing standards from the radioiodolipoprotein solutions with the same syringe and needle used to inject the tracer dose.

Definition of terms. For convenience, the following definitions are offered:

The *body pool* of a given plasma protein is the total amount of that protein in the body which is available for exchange with additional or newly synthesized molecules of that specific protein.

The *volume of distribution* of a protein is taken as the volume that a protein would occupy if the body pool of the protein had a uniform concentration equal to the plasma concentration of that protein. This term has *no anatomical implications* and does *not* indicate the extent of distribution of the protein in the body.

The *turnover half-time* of a plasma protein is the amount of time required for half of the specific protein molecules present in the body pool at a given moment to

TABLE I

Distribution of serum cholesterol and peptide among the lipoproteins in the individuals studied

Subject	Age (yrs.)	Total serum cholesterol (mg. %)	Cholesterol		
			β-lipoproteins		α-lipoproteins
			S_f 3 to 9	>S_f 9	(mg./fraction/100 ml. serum)
A. Normal adults					
J. B.	28	211	117	27	45
R. B.	29	221	124	38	32
D. C.	28				
W. H.	28				
J. K.	21				32
E. P.	29	190	100	27	40
F. R.	26		110	46	
B. S.	21				45
E. S.	26	178	93	46	24
B. Nephrotic children					
Group I					
K. S.	4 4/12	265	145	48	46
Group II					
E. T.	7 9/12	520	66	354	25
Group III					
J. G.	15 2/12	547	92	408	26
D. W.	4 4/12	980	64	774	15
K. W.	6 3/12	570	60	419	30
D. O.	6 1/12	884	141	624	22

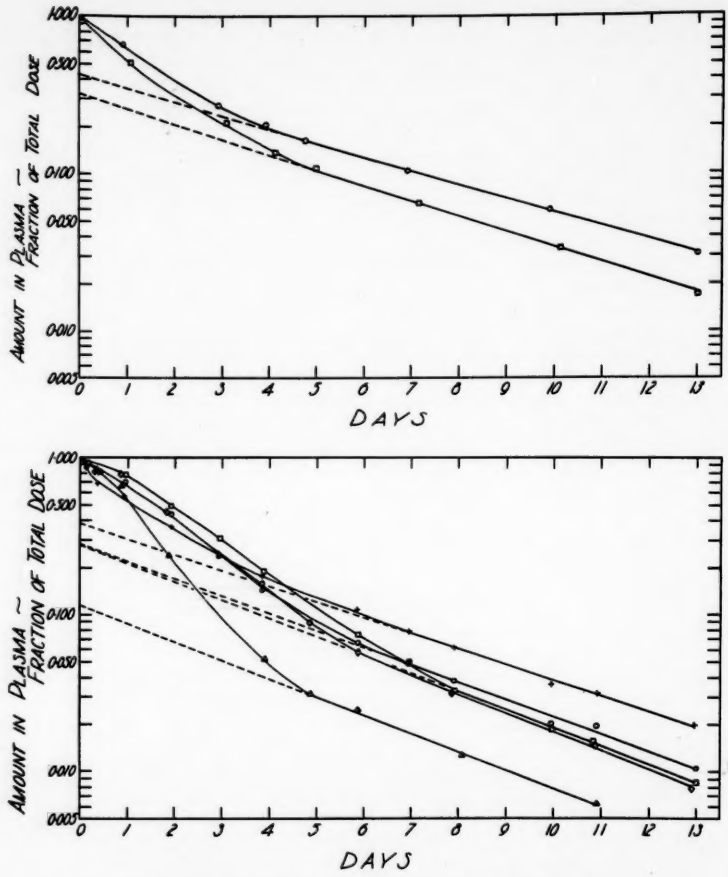

FIG. 2. THE DISAPPEARANCE OF IODINATED S_f 3 TO 9 LIPOPROTEINS FROM THE CIRCULATION AFTER INTRAVENOUS INJECTION

Upper chart, normal adults: □ J. B. ○ E. S.
Lower chart, nephrotic children: + K. S. □ E. T. ◇ J. G. ○ D. W. △ K. W.

be lost to the body through all channels of loss. The *turnover half-time due to catabolism* (or urinary loss) is the time required for half of the specific protein molecules present in the body pool at a given moment to be lost through catabolism (or urinary loss). The *fractional rate of turnover, catabolism, or urinary loss* is that fraction of the body pool of a given protein that is replaced, catabolized, or excreted, respectively, per unit time.

The values for lipoprotein turnover expressed in this paper are in terms of their peptide moieties, since the label is bound only to this portion of the lipoprotein molecule and the lipids may behave in an independent manner.

RESULTS

I. *Lipoprotein analyses*

The amount of cholesterol found in the different classes of lipoprotein present in the sera of the individuals studied are given in Table I. The values given are in terms of mg. of cholesterol per total lipoproteins of a given density class per 100 ml. of serum. It should be noted that in all of the children with hypercholesterolemia reported here, the serum elevation of cholesterol was attributable to an increase in the amount of low density lipo-

proteins; in these same children, the concentration of lipoprotein of S_f 3 to 9 was normal or decreased, as was the concentration of α-lipoproteins.

II. *Studies with radioiodinated serum lipoproteins of class S_f 3 to 9*

The disappearance of radioiodinated normal S_f 3 to 9 lipoproteins from the plasma of two normal adults and five children with the nephrotic syndrome is shown in Figure 2. Ultracentrifugal analysis of individual sera showed that 93 to 99 per cent of the iodinated S_f 3 to 9 lipoproteins retained in the plasma remained in the serum S_f 3 to 9 fraction throughout the period of study. The turnover half-times, as determined by simple graphic analysis of these curves, are indicated in Table II, A. It will be noted that the turnover half-times for this class of lipoproteins were only slightly less in the nephrotic children with hypercholesterolemia (Groups II and III) than in the normal subjects or in the child recovering from the nephrotic syndrome. When the curves of Figure 2 were corrected by the method of Berson, Yalow, Schreiber, and Post (22), the resulting turnover half-times were not significantly different from those obtained from the plasma disappearance curves alone. Examination of Figure 2 will reveal that the labeled lipoproteins disappeared from the plasma in an unusual fashion in all of the children with hypercholesterolemia; initially, the disappearance was gradual, then rapidly increased and finally became exponential. The reasons for the initial slow disappearance were not apparent from the data. Of the total injected dose of radioactivity, only 3 per cent or less appeared in the urine of the nephrotic children as labeled lipoprotein; in the normal individuals, none of the injected radioactivity was detected in the urine as labeled protein (Table II, A).

The volumes of distribution, as estimated by extrapolation of the single exponential phase of the plasma disappearance curves to zero time (Figure 2), seemed at first to be larger for these lipoproteins than has been the case for other plasma proteins studied in normal and nephrotic individuals (10). It has been shown, however, that such plasma disappearance curves cannot be

TABLE II

Turnover of S_f 3 to 9 lipoproteins and α-lipoproteins

Individual	Plasma volume (*ml.*)	Estimated cumulative urinary losses of iodinated protein* L_f*	Approximate half-time of turnover: From plasma disappearance $t_{\frac{1}{2}}$ (*days*)	Approximate half-time of turnover: Urinary loss $t_{e\frac{1}{2}}$ (*days*)	Approximate half-time of turnover: Catabolism $t_{e\frac{1}{2}}$ (*days*)	Fractional rate of turnover: Total λ_1 (*per day*)	Fractional rate of turnover: Urinary loss λ_e (*per day*)	Fractional rate of turnover: Catabolism λ_e (*per day*)
A. S_f 3 to 9 lipoproteins								
Normal								
J. B.	2,767	0.00	3.1	∞	3.1	0.224	0	0.224
E. S.	2,948	0.00	3.4	∞	3.4	0.204	0	0.204
Nephrotic								
I—K. S.	966	0.01	3.0	347	3.0	0.231	0.002	0.229
II—E. T.	1,294	0.01	2.6	231	2.6	0.267	0.003	0.264
III J. G.	1,071	0.03	2.5	87	2.6	0.277	0.008	0.269
III D. W.	789	0.01	2.8	347	2.8	0.248	0.002	0.246
III K. W.	625	0.01	2.5	231	2.5	0.277	0.003	0.274
B. α-lipoproteins								
Normal								
J. K.	3,238	0.00	4.8	∞	4.8	0.144	0	0.144
B. S.	3,493	0.00	4.4	∞	4.4	0.158	0	0.158
Nephrotic								
I—K. S.	995	0.01	3.7	347	3.7	0.187	0.002	0.185
II—E. T.	1,225	0.19	3.3	17	4.1	0.210	0.040	0.170
III—D. W.	804	0.25	2.3	9.2	3.1	0.301	0.075	0.226

* As fraction of total dose.

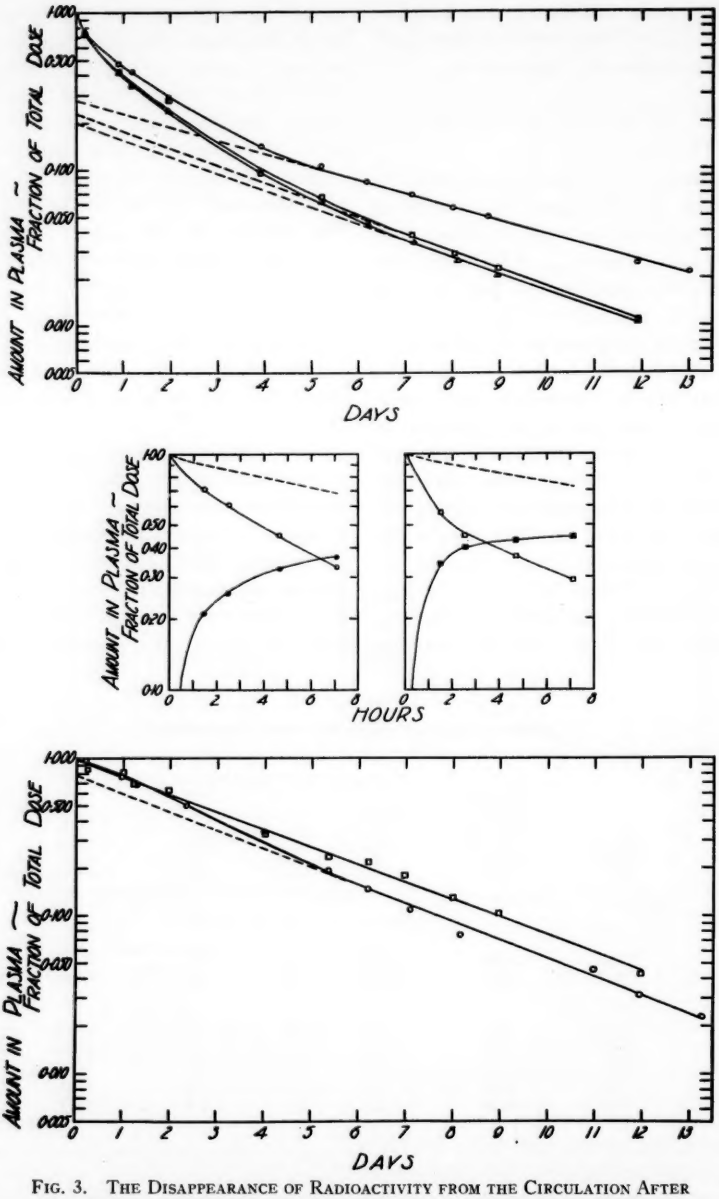

FIG. 3. THE DISAPPEARANCE OF RADIOACTIVITY FROM THE CIRCULATION AFTER INTRAVENOUS INJECTION OF IODINATED "NORMAL" LOW DENSITY LIPOPROTEINS

Upper chart, total plasma radioactivity in normal adults: □ J. P. △ R. B. ○ E. S.

Middle charts:

	W. H.	D. C.
Total plasma radioactivity	– – –	– – –
Low density lipoproteins	○	□
S_f 3 to 9 lipoproteins	●	■

Lower chart, total plasma radioactivity in nephrotic children: ○ J. G. □ D. O.

used directly for estimation of the volume of distribution of a body pool of endogenous protein, particularly in an edematous child. The volumes of distribution for endogenous plasma proteins determined by simple extrapolation of these curves would be overestimated to a degree reflected by: *1*) the higher fractional rate of catabolism of labeled protein prior to establishment of the steady state (*22*), and *2*) the higher specific activities of labeled protein present extravascularly compared to the specific activities in the plasma during the steady state (10). Estimation of the volume of distribution by the method of Berson, Yalow, Schreiber, and Post (*22*) tends to correct the plasma disappearance curves for the first factor, *i.e.*, initial variations in the fractional rate of catabolism of labeled protein. Using the urinary excretion data and employing this method, the volumes of distribution of S_f 3 to 9 lipoproteins in the two normal subjects, J. B. and E. S., and in the child in Group I, K. S., were, respectively, 2.22, 2.27, and 2.17 times the plasma volumes; the plasma compartment contained 45 per cent, 44 per cent, and 46 per cent of the total body pool,

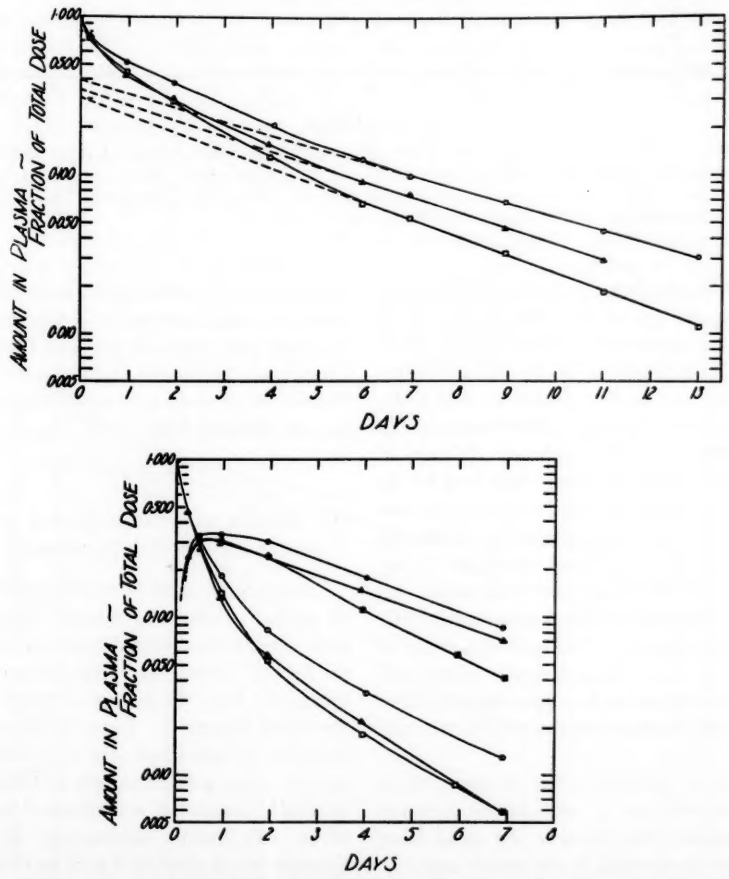

FIG. 4. THE DISAPPEARANCE OF RADIOACTIVITY FROM THE CIRCULATION AFTER INTRAVENOUS INJECTION OF IODINATED "NEPHROTIC" LOW DENSITY LIPOPROTEINS IN NORMAL ADULTS

Upper chart, total plasma radioactivity: △ R. B. □ E. P. ○ F. R.

Lower chart: open symbols indicate low density lipoproteins and solid symbols indicate lipoproteins of S_f 3 to 9 in same subjects as upper chart.

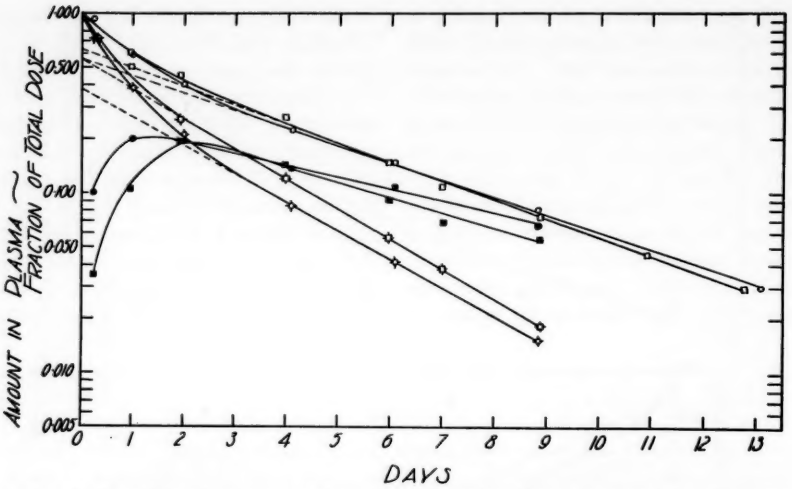

FIG. 5. THE DISAPPEARANCE OF TOTAL RADIOACTIVITY (OPEN SYMBOLS), LOW DENSITY LIPOPROTEINS (STARRED SYMBOLS) AND S_f 3 TO 9 LIPOPROTEINS (SOLID SYMBOLS) FROM THE CIRCULATION AFTER INTRAVENOUS INJECTION OF IODINATED "NEPHROTIC" LOW DENSITY LIPOPROTEINS IN TWO NEPHROTIC CHILDREN
○ J. G. □ D. O.

respectively. Where there are marked differences in the specific activity of the labeled protein in different body compartments, however, or where there is significant retention of the labeled degradation products, this method cannot be used (10). Thus, the calculated volumes of distribution for S_f 3 to 9 lipoproteins in the nephrotic children of Group III were larger than was indicated by direct analyses of edema and ascitic fluids; the concentration of S_f 3 to 9 lipoproteins in edema and ascitic fluids of these children, estimated by ultracentrifugal fractionation and cholesterol determinations, was found to be approximately 0.01 times that in the plasma. The specific activity of the iodinated S_f 3 to 9 lipoproteins in edema and ascitic fluids was found to be approximately three times that in the plasma after the steady state was established.

In Table II, A, are shown the calculated fractional rates of turnover as well as the turnover half-times obtained from them in the usual manner. It can be shown that, in the steady state,

$$L_f^* = \frac{\lambda_e}{\lambda_t}, \qquad 1)$$

where L_f^* is that fraction of the total dose of iodinated lipoprotein lost in the urine as labeled protein, λ_e is the fraction of the body pool lost in the urine per unit time and λ_t is the total fraction of the body pool replaced per unit time; λ_t was obtained from the plasma disappearance curves. The fraction of the body pool catabolized per unit time, λ_c, was obtained from

$$\lambda_c = \lambda_t - \lambda_e. \qquad 2)$$

III. *Studies with radioiodinated serum lipoproteins of low density (S_f values of 10 to 200)*

Lipoproteins of S_f 10 to 100, isolated from sera of normal adults and labeled with radioiodine, were given to five normal adults and two nephrotic children of Group III. The disappearance of radioactivity from the plasma of these individuals is shown in Figure 3. Upon ultracentrifugal fractionation of individual sera at various times during the study, it was apparent that the radioactivity originally associated with lipoproteins of class S_f 10 to 100 became increasingly associated with lipoproteins of class S_f 3 to 9 as time progressed. Unfortunately, only random sera of these individuals were fractionated, except for two normal adults shown in Figure 3.

The overall behavior of lipoproteins of class S_f 10 to 200 obtained from a nephrotic child and la-

beled with radioiodine was similar to, but not identical with, that of the labeled "normal" lipoproteins of S_f 10 to 100. The labeled "nephrotic" low density lipoproteins were given to three normal adults and the same two children of Group III. The disappearance of total radioactivity from the plasma of these subjects and the relative amount of radioactivity in the different lipoprotein fractions were determined; the results are indicated in Figures 4 and 5. The initial, rapidly falling portion of the plasma disappearance curves for the low density lipoproteins was attributable to at least three factors: *1*) diffusion from the vascular compartment, *2*) conversion to higher density β-lipoproteins, and *3*) catabolism. Later, the phase of steady logarithmic decline was presumably mainly due to the last two factors. In the nephrotic children, less than 3 per cent of the total dose was lost in the urine as lipoproteins, but the classes of lipoproteins that this represented could not be determined. It will be noted that the conversion of low density lipoproteins to S_f 3 to 9 lipoproteins was decreased in the nephrotic children compared to the normal adults; in the normal adults, labeled S_f 3 to 9 lipoproteins appeared more rapidly and reached a higher level than in the nephrotic children.

The volume of distribution of the low density lipoproteins could not be accurately determined from the plasma disappearance curves. In addition to the factors mentioned in the case of the lipoproteins of S_f 3 to 9, there was the additional complication of a rapid conversion of low density lipoproteins to those of S_f 3 to 9 in the normal adults. The situation in the *normal* subjects was analogous to that described for albumin in nephrotic children (10) where an equally rapid loss to the vascular pool of a specific protein occurred through urinary loss instead of by conversion. It was reported that under these circumstances the estimated volumes of distribution were several-fold too large and the estimated half-times of turnover were too long.

IV. *Studies with radioiodinated α-lipoproteins*

Radioiodinated normal α-lipoproteins were given to two normal adults and three children with the nephrotic syndrome (Table II, B). The disappearance of these lipoproteins from the plasma of these individuals is shown in Figure 6. Ultra-

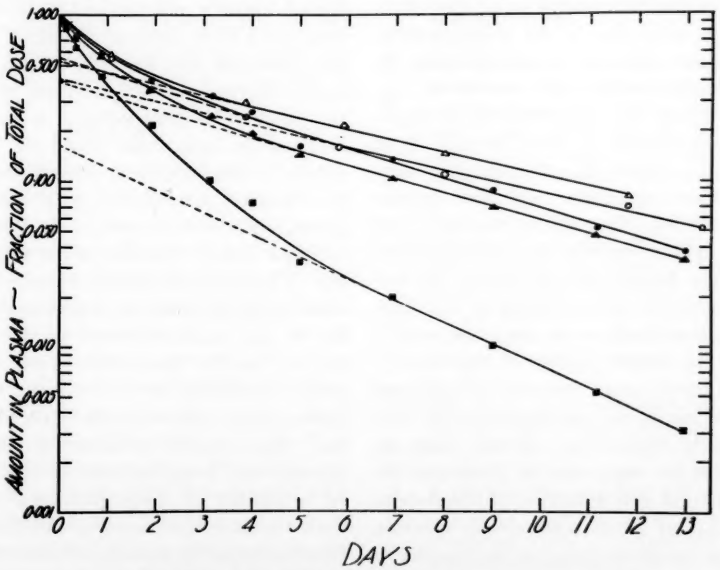

FIG. 6. THE DISAPPEARANCE OF IODINATED α-LIPOPROTEINS FROM THE CIRCULATION AFTER INTRAVENOUS INJECTION

△ J. K. ○ B. S. ● K. S. ▲ E. T. ■ D. W.

centrifugal fractionation of the sera did not reveal any significant shift of radioactivity to chylomicra or to any of the other lipoprotein fractions.

The two normal adults revealed no detectable iodinated lipoprotein in the urine; the child of Group I lost only 1 per cent of the total dose in the urine as protein. The volumes of distribution of α-lipoproteins in these three persons averaged 2.0 times the plasma volume when calculated according to the method of Berson, Yalow, Schreiber, and Post (*22*). The two children of Groups II and III, E. T. and D. W., excreted, respectively, 19 and 25 per cent of the total dose of labeled α-lipoproteins in the urine as labeled protein. The turnover of α-lipoproteins in these patients as calculated from equations *1*) and *2*) is shown in Table II, B. In the case of D. W., where a relatively large urinary loss of labeled protein occurred in a child with anasarca and ascites, the turnover half-time as estimated from the plasma disappearance curve represents a maximum value (10); therefore, the derived turnover half-time due to catabolism also represents a maximum value.

DISCUSSION

It is apparent that the metabolism of the α-lipoproteins differs from that of the β-lipoproteins. After intravenous injection of α-lipoproteins labeled in the peptide moiety with radioiodine, significant radioactivity was not detected in chylomicra or in lipoproteins of lower density or of slower mobility. Similarly, radioactivity originally injected in association with the peptide moiety of the labeled β-lipoproteins was not found subsequently in α-lipoproteins or in chylomicra. In contrast, the labeled peptide moiety of low density β-lipoproteins rapidly appeared in β-lipoproteins of higher density soon after intravenous injection, but the labeled peptide of high density β-lipoproteins could not be detected in significant amounts in the low density lipoproteins even after several weeks *in vivo*. The evidence does not seem to support the suggestion of Korn that the peptide moieties of α-lipoproteins, β-lipoproteins and chylomicra are intimately related, entering into a common metabolic cycle for the transfer of lipids (9). Instead, the findings suggest that, in humans at least, the metabolism of the peptide moiety of α-lipoprotein is independent of the metabolism of the peptide moiety of β-lipoprotein and that there is no direct conversion of α- or β-lipoprotein in the formation of chylomicra. It is not possible to determine from our data whether the peptide portion of chylomicra ever becomes that of either the α- or β-lipoproteins, or if it does occur, whether such conversion is quantitatively significant.

This concept of lipoprotein metabolism is in accord with immunochemical studies of the lipoproteins. Although the β-lipoproteins are heterogeneous as regards *quantitative* precipitation with specific antisera, the haptenic groups of the peptide moieties of the β-lipoproteins studied are at least *qualitatively* similar irrespective of the density of the β-lipoprotein molecule (23–25). Moreover, antisera against β-lipoproteins do not react with α-lipoproteins.

The metabolism of α-lipoproteins in the nephrotic syndrome is similar to that found for other plasma proteins such as albumin or γ-globulin (10); in the full-blown disease, the fractional rate of catabolism of α-lipoproteins is increased.

In the children reported here, hypercholesterolemia was due to an elevation of the plasma concentration of low density lipoproteins. This is in accord with the observations of Havel, Eder, and Bragdon (3) in nephrotic adults. It would appear from the data that this finding is associated with a decrease in the conversion of the low density lipoproteins to lipoproteins of S_f 3 to 9. This decrease in conversion could be attributed to either: *1*) an increase in plasma concentration of low density lipoproteins with saturation of a normal conversion system, or *2*) a decrease in one or more factors essential to the conversion process. The second possibility is compatible with the observation that albumin is necessary for the binding of fatty acids liberated by the hydrolysis of neutral fats by lipoprotein lipase (*26*); an absence of albumin or a deficiency of lipoprotein lipase in the system results in decreased hydrolysis. The possible relationship between hyperlipemia and hypoalbuminemia has been studied by a number of investigators (*27–30*). It has been reported that repeated infusions of concentrated albumin in patients with the nephrotic syndrome resulted in a fall in plasma cholesterol (30) and, similarly, that albumin infusions in nephrotic rats reduced cholesterol and total lipids (*27*).

It has also been observed, however, that children with the nephrotic syndrome show varying degrees of lipoprotein lipase deficiency (31); Rosenman and Friedman have recently reported similar findings in nephrotic rats (32). It has been found that in some nephrotic children, hypercholesterolemia was due primarily to an elevation of lipoproteins of S_f 3 to 9, but, in these instances, the S_f 10 to 200 lipoproteins were also elevated (33, 34).

It has been implicitly assumed in this study that the iodinated lipoproteins behaved metabolically as did the native proteins. However, some of the results obtained are not readily explained. Careful examination of the middle graph of Figure 3 suggests some heterogeneity in the labeled low density lipoproteins with respect to conversion; whether this was the result of partial denaturation due to iodination or an inherent heterogeneity in the conversion rates of low density lipoproteins could not be determined. It will also be noted that the initial plasma disappearance of total radioactivity from iodinated "normal" low density lipoproteins in the nephrotic children (bottom graph, Figure 3) appeared to be slower than that obtained with analogous "nephrotic" lipoproteins in the same children (Figure 5). It is not known whether this was due to an actual difference in the native lipoproteins, a difference in the state of the children, or a difference induced by iodination.

SUMMARY

Ultracentrifugal fractionation of sera from six normal adults and six children in various phases of the nephrotic syndrome revealed that hypercholesterolemia and hyperlipemia in the nephrotic syndrome are associated with an elevation of low density lipoproteins, those of S_f 10 to 200. Serum concentrations of lipoproteins of S_f 3 to 9 and of α-lipoproteins were relatively normal or somewhat decreased in the nephrotic children studied.

The peptide moieties of α-lipoproteins, lipoproteins of S_f 3 to 9 and lipoproteins of S_f 10 to 100 from normal adults and of S_f 10 to 200 lipoproteins from a nephrotic child were labeled with I^{131}. Tracer doses of these iodolipoproteins were given intravenously to children with the nephrotic syndrome and to normal individuals. The disappearance of specific radioiodinated protein from the plasma and the appearance of radioactivity in other plasma protein fractions and in the urine were investigated.

It was found that the fractional rate of catabolism of α-lipoprotein was somewhat increased in a nephrotic child with anasarca and ascites. In two nephrotic children, 19 per cent and 25 per cent, respectively, of the tracer dose of radioiodinated α-lipoproteins were excreted in the urine as labeled protein.

The fractional rate of catabolism of S_f 3 to 9 lipoproteins tended to be somewhat increased in the nephrotic patients. Less than 3 per cent of the total dose of S_f 3 to 9 iodolipoproteins appeared in the urine as labeled protein in those children with significant proteinuria.

The metabolism of the *low density lipoproteins* was complex. In both normal and nephrotic persons, a significant proportion of the S_f 10 to 200 iodolipoproteins was converted to those of S_f 3 to 9. In the nephrotic children, the conversion of low density lipoproteins was decreased.

REFERENCES

1. Oncley, J. L. Lipoproteins of human plasma. Harvey Lect. 1954–1955, **50**, 71.
2. Kunkel, H. G., and Trautman, R. The α_2 lipoproteins of human serum. Correlation of ultracentrifugal and electrophoretic properties. J. clin. Invest. 1956, **35**, 641.
3. Havel, R. J., Eder, H. A., and Bragdon, J. H. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. clin. Invest. 1955, **34**, 1345.
4. Lindgren, F. T., Nichols, A. V., and Freeman, N. K. Physical and chemical composition studies on the lipoproteins of fasting and heparinized human sera. J. phys. Chem. 1955, **59**, 930.
5. Oncley, J. L., and Mannick, V. G. Studies on the human plasma lipoproteins. Trans. Fifth int. Congr. on Blood Transfusion, Paris 1954, p. 116.
6. Oncley, J. L., Walton, K. W., and Cornwell, D. G. A rapid method for the bulk isolation of β-lipoproteins from human plasma. J. Amer. chem. Soc. In press.
7. Gould, R. G. Lipid metabolism and atherosclerosis. Amer. J. Med. 1951, **11**, 209.
8. Kunkel, H. G., and Bearn, A. G. Phospholipid studies of different serum lipoproteins employing P-32. Proc. Soc. exp. Biol. (N. Y.) 1954, **86**, 887.
9. Korn, E. D. Clearing factor, a heparin-activated lipoprotein lipase. II. Substrate specificity and activation of coconut oil. J. biol. Chem. 1955, **215**, 15.
10. Gitlin, D., Janeway, C. A., and Farr, L. E. Studies on the metabolism of plasma proteins in the ne-

phrotic syndrome. I. Albumin, γ-globulin and iron-binding globulin. J. clin. Invest. 1956, **35**, 44.

11. Randolph, M. L., and Ryan, R. R. A slicer for sampling liquids. Science 1950, **112**, 528.
12. de Lalla, O. F., and Gofman, J. W. Ultracentrifugal analysis of serum lipoproteins *in* Methods of Biochemical Analysis, D. Glick, Ed. New York, Interscience Publishers, 1954, vol. 1, p. 459.
13. Williams, J. W., Saunders, W. M., and Cicirelli, J. S. Size distribution analysis in plasma-extender systems. I. Gelatin. J. phys. Chem. 1954, **58**, 774.
14. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K., and Uroma, E. A system for the separation of the components of human blood: Quantitative procedures for the separation of the protein components of human plasma. J. Amer. chem. Soc. 1950, **72**, 465.
15. Hillyard, L. A., Entenman, C., Feinberg, H., and Chaikoff, I. L. Lipid and protein composition of four fractions accounting for total serum lipoproteins. J. biol. Chem. 1955, **214**, 79.
16. Latta, H. Experimental hypersensitivity in the rabbit. Blood and tissue concentrations of foreign proteins labeled with radioactive iodine and injected intravenously. J. Immunol. 1951, **66**, 635.
17. Pressman, D., and Eisen, H. N. The zone of localization of antibodies. V. An attempt to saturate antibody-binding sites in mouse kidney. J. Immunol. 1950, **64**, 273.
18. Ray, B. R., Davisson, E. O., and Crespi, H. L. Experiments on the degradation of lipoproteins from serum. J. phys. Chem. 1954, **58**, 841.
19. Yalow, R. S., and Berson, S. A. Chemical and biological alterations induced by irradiation of I^{131} labeled human serum albumin. J. clin. Invest. 1957, **36**, 44.
20. Kunkel, H. G., and Slater, R. J. Zone electrophoresis in a starch supporting medium. Proc. Soc. exp. Biol. (N. Y.) 1952, **80**, 42.
21. Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E. A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. biol. Chem. 1952, **195**, 357.
22. Berson, S. A., Yalow, R. S., Schreiber, S. S., and Post, J. Tracer experiments with I^{131} labeled human serum albumin: Distribution and degradation studies. J. clin. Invest. 1953, **32**, 746.
23. Gitlin, D. The immunochemical heterogeneity of human plasma β-lipoprotein. Science 1953, **117**, 591.
24. Levine, L., Kauffman, D. L., and Brown, R. K. The antigenic similarity of human low density lipoproteins. J. exp. Med. 1955, **102**, 105.
25. Aladjem, F., Lieberman, M., and Gofman, J. W. Immunochemical studies on human plasma lipoproteins. J. exp. Med. 1957, **105**, 49.
26. Nikkilä, E. A., and Hashti, E. On the mechanism of the heparin-induced lipemia clearing reaction. Acta chem. scand. 1954, **8**, 363.
27. Rosenman, R. H., Friedman, M., and Byers, S. O. The causal role of plasma albumin deficiency in experimental nephrotic hyperlipemia and hypercholesteremia. J. clin. Invest. 1956, **35**, 522.
28. Thomas, E. M., Rosenblum, A. H., Lander, H. B., and Fisher, R. Relationships between blood lipid and blood protein levels in the nephrotic syndrome. Amer. J. Dis. Child. 1951, **81**, 207.
29. Gurin, S. Discussion of paper by Langdon, R. G. Cholesterol metabolism *in* Fat Metabolism, Report of the Eleventh M & R Pediatric Research Conference, Johns Hopkins Hospital. Columbus, M & R Laboratories, 1954, p. 42.
30. Soothill, J. A., and Kark, R. M. The effects of infusion of salt-poor human serum albumin on serum cholesterol cholinesterase, and albumin levels in healthy subjects and in patients ill with the nephrotic syndrome. Clin. Res. Proc. 1956, **4**, 140.
31. Gitlin, D., and Gross, P. A. M. The mechanism of hyperlipemia in the nephrotic syndrome. Trans. Soc. Pediat. Res. May, 1957.
32. Rosenman, R. H., and Friedman, M. *In vivo* studies of the role of albumin in endogenous and heparin-activated lipemia-clearing in nephrotic rats. J. clin. Invest. 1957, **36**, 700.
33. Gitlin, D., Gross, P. A. M., and Numa, S. Unpublished data.
34. Baxter, J. H., Goodman, H. C., and Havel, R. J. Hyperlipemia of nephrosis (abstract). J. clin. Invest. 1957, **36**, 873.

THE FUNCTIONAL CAPACITY OF THE KIDNEY DENERVATED BY AUTOTRANSPLANTATION IN THE DOG [1]

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The influence of renal nerves on the renal tubular transport of sodium chloride has been a source of controversy for many years. Both conceptual and methodologic differences have contributed to the continuing conflict. The conceptual disagreements concern the primacy of glomerular filtration rate changes (as opposed to intrinsic alteration in tubular transport) in explaining differing rates of sodium excretion between denervated and innervated kidneys in the same animals. The methodologic problems relate to the validity of the procedures conventionally employed for denervation. The first of these, interruption of all visible nerves entering at the hilus, has been criticized because of the possibility that some nerve fibers may be left intact. The second, section of the splanchnic nerves, has been criticized because splanchnic structures other than the kidneys lose their nerve supply. The contention of Quinby (1) that complete denervation depends upon section and resuture of renal artery, vein and ureter has been frequently cited (2).

Studies have recently been reported on a human kidney which fulfilled Quinby's criteria for denervation (3). This organ, which had been transplanted from one identical twin to another, was totally denervated at the time of transplantation, and the denervation was restricted to the involved organ. The objections to conventionally denervated kidneys were thereby obviated. The data demonstrated that the transplanted kidney could accomplish the majority of functions examined in a normal or near-normal manner. However, no definitive conclusions regarding the role of renal nerves on sodium transport could be drawn. The present studies were initiated in order to further explore this problem, as well as to gain additional information about the functional capabilities of transplanted kidneys.

Experiments have been performed on dogs in which one kidney was autotransplanted from the renal fossa to the iliac fossa. The opposite kidney in each animal served as the control organ. Individual functions of both kidneys were examined simultaneously at intervals, both before and after transplantation. Similar studies were performed on two dogs in which one kidney was denervated conventionally. Comparison of the experiments on the kidney conventionally denervated with those on the transplanted kidney has provided a means of evaluating the former as an experimental model.

METHODS

Studies were performed on eight female mongrel dogs in which the urinary bladder was divided longitudinally between the ureteral orifices. Each half was then fashioned into a discrete hemibladder (4). The two kidneys of each animal were thereby provided with individual bladders drained by permanent polyethylene tubes protruding through stab wounds in the anterior abdominal wall. With proper care to prevent plugging of the catheters, such animals have been found to remain healthy and vigorous indefinitely. With one exception, each animal was subjected to from one to three preliminary clearance studies (prior to denervation) in order to define the functional interrelationships of the two kidneys. In six dogs, one kidney was autotransplanted (and thereby denervated) from the renal fossa to the iliac fossa using a procedure similar to that described by Murray, Lang, Miller, and Dammin (5). The ureter of each transplanted kidney was reimplanted into its own hemibladder. In two dogs denervation was accomplished by stripping the adventitia from the renal vessels and dissecting the kidney from adherent and adjacent tissue

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which might contain renal nerves. The contralateral kidney in each animal was left intact to serve as the control organ. Subsequent to the surgical procedure, from one to four follow-up studies were performed. The earliest of these was eight days postdenervation and the latest 163 days postdenervation. Thirteen of the 18 postdenervation experiments were made within 23 days of denervation.

Standard renal clearance procedures were employed. Glomerular filtration rate (GFR) was determined by exogenous creatinine clearance (C_{Cr}) and effective renal plasma flow (ERPF) by *p*-aminohippurate clearance (C_{PAH}). The animals were studied in the unanesthetized state and in the standing position, supported by an abdominal sling with openings for the limbs and catheters. The height of the sling was adjusted so as to allow the legs to touch the base of a table.

Urine collection periods were concluded with water and/or air injections (0.5 ml.), using 2 ml. syringes which adapted to the catheters. Heparinized venous blood samples were drawn at the mid-point of each period through an inlying polyethylene catheter in the external jugular vein. Because of the necessity for removing large volumes of blood on multiple occasions, the red cells from each blood sample were resuspended in physiologic saline, filtered through sterile gauze and reinfused. The analytic methods employed have been described previously (*3*).

Three types of experiments were principally employed to examine electrolyte excretion: *1*) Sustained isotonic expansion of extracellular fluid (ECF) volume was induced by the infusion of a simulated ECF solution at 8.8 ml. per minute. The total volume of infusate was calculated to expand ECF by 25 to 50 per cent. In order to maintain the expanded volume, as well as to preserve the constancy of plasma Na and Cl concentrations, the estimated urinary losses of sodium, chloride and water were replaced throughout the experiment by the infusion of synthetic urine solutions as described by Wesson, Anslow, Raisz, Bolomey, and Ladd (*6*). During the final three clearance periods (after the completion of expansion), 50 mμ of vasopressin (Pitressin®) was injected as a priming dose and 1 mμ per minute was delivered in the sustaining infusion of creatinine and PAH. *2*) Hypertonic expansion of ECF volume was induced by the infusion of three per cent saline at 5 ml. per minute. In one study, ECF volume was expanded with hypotonic saline. *3*) Anesthesia was induced by the intravenous administration of sodium pentobarbital (30 mg. per Kg.) after the third clearance period.

In two studies, sodium excretion was examined following the intravenous administration of mercurial diuretics. In the majority of experiments, the first three clearance periods were control periods and were obtained under conditions of a diuresis induced by the previous administration of water (20 to 30 ml. per Kg.) by stomach tube.

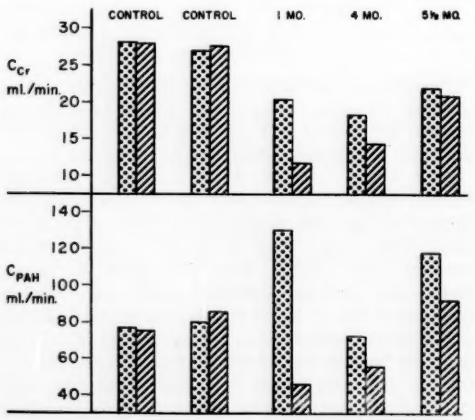

FIG. 1. SERIAL CHANGES IN BILATERAL RENAL HEMODYNAMICS FOLLOWING UNILATERAL AUTOTRANSPLANTATION

Stippled bars represent intact kidney, cross-hatched bars transplanted kidney. Control studies were performed before unilateral transplantation.

RESULTS

I. *Transplanted kidneys*

Renal hemodynamics. In the initial studies following transplantation, values for C_{Cr} and C_{PAH} for the transplanted kidneys were appreciably below their respective control levels. The changes varied from a 44 per cent decrease to almost complete suppression of function in one dog. In one of the six animals, clearance values were higher for the transplanted than the intact kidney; but unfortunately, this was the single dog in which no control studies were available. In most animals, reciprocal changes in clearance values occurred in the intact kidney, with C_{PAH} increasing proportionally more than C_{Cr}. Filtration fractions typically decreased bilaterally and equally. In the transplanted kidneys, this change resulted from a greater *decrease* in C_{Cr} than C_{PAH}, whereas in the intact kidneys it represented a greater *increase* in C_{PAH} than C_{Cr}.

In the three dogs in which serial studies were performed following transplantation, clearance values increased with time. In two of these the values for the two kidneys approached equality. In Figure 1 data are shown for an animal followed for 163 days subsequent to autotransplantation.

The transplanted kidney maintained its capacity to increase filtration rate and renal plasma

TABLE I

Simultaneous effects of volume expansion on the glomerular filtration rate in transplanted and intact kidneys

Dog	Day after transplant	Clearance period	Infusate	C_{Cr}* ml./min. TK†	C_{Cr}* ml./min. IK‡	Per cent increase from mean control value TK	Per cent increase from mean control value IK
RQ	20	1–3 (mean)	0.9% NaCl at 1.5 ml./min.	20.9	44.5		
		4	Isotonic (simulated ECF)	20.8	39.9		
		5	solution at 8.8 ml./min.	19.2	45.6		2.5
		6		19.2	41.8		
		7		21.9	51.4	4.8	15.5
		8		28.4	54.1	35.9	21.6
		9		29.4	64.6	40.7	45.2
		10		32.9	60.2	57.4	35.3
		11		29.3	53.4	40.2	19.8
		12		26.6	57.8	27.3	29.9
		13		18.3	50.0		12.4
LG	56	1–3 (mean)	0.9% NaCl at 1.5 ml./min.	4.4	16.1		
		4	3% NaCl at 5.0 ml./min.	6.8	23.2	54.5	44.1
		5		6.7	24.5	52.3	52.2
		6		7.8	26.9	77.3	67.1
		7		7.8	27.2	77.3	68.9
		8		9.2	30.3	109.1	88.2

* C_{Cr} = Creatinine clearance.
† TK = Transplanted kidney.
‡ IK = Intact kidney.

flow during ECF expansion regardless of the absolute level of these functions at the time of the study. Moreover, the increases in clearance values for the transplanted kidneys were generally proportional to those noted for the intact kidneys. That these changes were a function of volume expansion rather than the particular infusate employed is suggested by the similarity of responses noted following the infusion of hypotonic, isotonic and hypertonic solutions. Two representative experiments are shown in Table I.

Following the induction of anesthesia, the GFR in normally innervated kidneys often decreases. In kidneys denervated by conventional methods, this anticipated decrease in clearance values has frequently been absent. The results of induction of anesthesia with intravenous sodium pentobarbital, after three control periods, in a dog with one kidney autotransplanted are shown in Figure 2. The intact (normally innervated) kidney sustained a decrease in C_{Cr}, whereas the values for the transplanted kidney remained essentially constant. This response was not invariable, however, and in Table II the results of an experiment in which GFR decreased relatively more for the transplanted than intact kidney are shown.

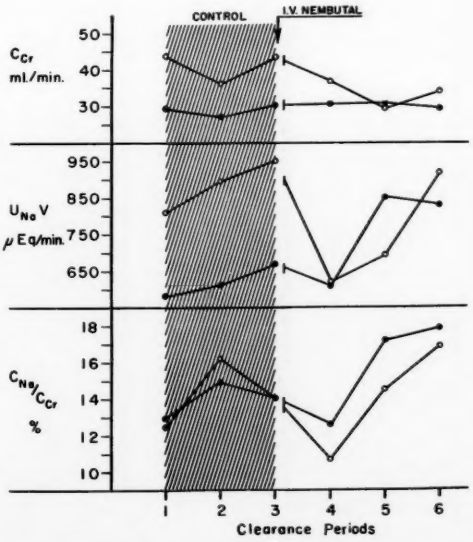

FIG. 2. EFFECTS OF ANESTHESIA BILATERALLY FOLLOWING UNILATERAL TRANSPLANTATION

Open circles represent intact kidney, closed circles transplanted kidney. $U_{Na}V$ refers to minute rate of Na excretion and C_{Na}/C_{Cr} to percentage of filtered sodium excreted. Donnan factor for sodium was 0.95.

TABLE II

Effects of anesthesia on the creatinine clearance, sodium and potassium excretion rates during 3 per cent saline infusion in transplanted and intact kidneys

Dog	Day	Clearance period	C_{Cr} (*ml./min.*) TK‡	C_{Cr} (*ml./min.*) IK§	C_{Cr} TK‡/IK§	$U_{Na}V$* (*μEq./min.*) TK‡	$U_{Na}V$* (*μEq./min.*) IK§	Per cent FL† Na excreted TK‡	Per cent FL† Na excreted IK§	Per cent FL† Na excreted TK‡/IK§	U_KV† (*μEq./min.*) TK‡	U_KV† (*μEq./min.*) IK§	U_KV† TK‡/IK§
PW	21	1	20.8	11.7	1.78	118	77	4.2	4.8	0.88	28.2	11.1	2.54
		2	23.1	16.2	1.43	209	176	6.4	7.3	0.88	47.5	28.2	1.68
		3	21.6	14.2	1.52	273	245	8.1	11.5	0.70	36.5	28.6	1.28
		4	20.4	14.0	1.46	294	244	9.1	10.9	0.83	31.7	24.3	1.30
	Mean	1–4	21.5	14.0	1.54	223	186	7.0	8.6	0.81	36.0	23.1	1.56
			Intravenous administration of Na pentobarbital (30 mg./Kg.)										
		5	14.5	12.8	1.13	96	138	3.8	6.2	0.61	12.6	12.9	0.98
		6	21.3	13.8	1.54	243	215	6.6	9.0	0.73	28.9	12.5	2.31
		7	19.3	12.8	1.51	278	308	8.1	13.6	0.60	20.1	20.0	1.01
	Mean	5–7	18.4	13.1	1.40	206	220	6.2	9.6	0.65	20.5	15.1	1.36
Per cent change in mean values			−14.4	−6.4		−7.5	+18.2	−11.4	+11.6		−43	−34.6	

* UV = Minute rate of excretion.
† FL = Filtered load.
‡ TK = Transplanted kidney.
§ IK = Intact kidney.

Concentration and dilution. Values for free water clearance (C_{H_2O}) during isotonic expansion of ECF volume are shown in Figure 3. A progressive increase occurred for both the transplanted and the intact kidneys. Following the initiation of Pitressin® infusion, C_{H_2O} fell bilaterally. When values for free water clearance are factored by the respective values for filtered water (lower half of Figure 3), the ratios for the two kidneys approach equality and remain comparable during Pitressin® infusion. In a similar experiment on a different dog, the urine from both kidneys became concentrated during the infusion of Pitressin®. The values for solute free water abstracted ($T^c_{H_2O}$) were numerically greater for the intact kidney, but so also was the filtration rate, and the ratios $T^c_{H_2O}/C_{Cr}$ were equal for the two kidneys.

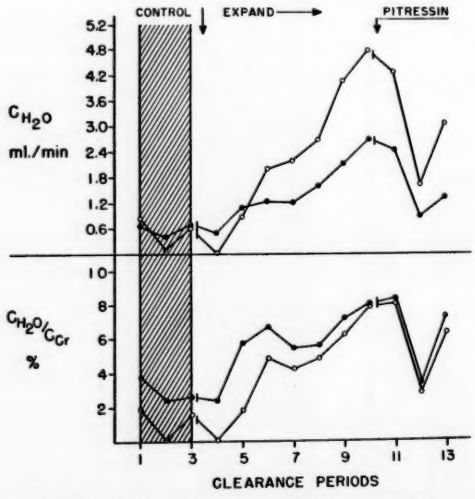

FIG. 3. FREE WATER CLEARANCE DURING ECF EXPANSION THREE WEEKS POST-TRANSPLANT

Open circles represent intact kidney, closed circles transplanted kidney. Each point is the value of a 15 to 30 minute clearance period. C_{H_2O} refers to free water clearance.

Sodium excretion. Values for sodium excretion rates during the control clearance periods (fasting state, without ECF expansion) were greater in some experiments for the transplanted kidneys (despite lower filtration rates) than for the control kidneys. This was not a consistent finding, however, either for the group or in serial studies on individual dogs (Table III) and in view of the difficulties in interpretation of data obtained during low rates of sodium excretion, no functional significance has been ascribed to these findings.

The infusion of hypertonic saline has been

widely employed in studies designed to examine the function of denervated kidneys.[4] Several investigators have found that during such experiments, the denervated kidney excretes a higher percentage of its filtered load of sodium than the contralateral kidney (8, 9). In Table IV-A, results of experiments on four dogs with unilaterally transplanted kidneys are shown. Fortuitously, in two of these experiments, filtration rates of the transplanted kidneys were greater than those of the intact organs, and in the other two, the intact kidneys had the greater filtration rates. In none of these animals did the transplanted kidney excrete a greater percentage of filtered sodium than the intact kidney. Thus, in one dog (SP) comparable values were obtained for the two kidneys and in three dogs the values were *less* for the transplanted than the intact kidneys.

When anesthesia was induced during the course of a brisk sodium diuresis attendant upon hypertonic saline infusion, deviant responses in sodium excretion by the two kidneys appeared to be mediated by the respective changes in filtration rate. The plasma sodium levels generally increased throughout such experiments. This term in the filtered load expression, therefore, contributed to a progressive increase in sodium excretion by both kidneys and magnified the effects of small changes in filtration rate on sodium excretion rates. When the GFR of the intact kidney decreased more than that of the transplanted kidney (Figure 2), the percentage of filtered sodium excreted became proportionally greater for the transplanted kidneys. When, however, the GFR decreased more for the transplanted than for the intact kidney (Table II) the opposite relationship was noted.

Expansion of extracellular fluid volume isotonically without changing Na or Cl concentrations permits the examination of sodium *reabsorption* (during high rates of sodium excretion) where changes in filtered load are due entirely to changes in the GFR. Comparison of the response of transplanted kidneys and intact kidneys during

[4] A detailed review of the pertinent literature may be found in the recent review by Wesson (7).

TABLE III

Bilateral values for the glomerular filtration rate, sodium and potassium excretion rates in the fasting state without extracellular fluid expansion (control clearance periods) before and after unilateral transplantation

Dog	Day after transplant	C_{Cr} ml./min.			$U_{Na}V$ μEq./min.			U_KV μEq./min.		
		TK*	IK†	TK*/IK†	TK*	IK†	TK*/IK†	TK*	IK†	TK*/IK†
RQ	Pre‡	38.1	39.2	0.97	0.67	1.0	0.67	5.7	7.1	0.80
	Pre	28.8	35.8	0.80	0.63	1.0	0.63	3.1	2.9	1.07
	20	20.9	44.5	0.46	6.2	2.0	3.1	7.4	15.5	0.48
SP	Pre	28.4	28.3	1.0	4.4	4.7	0.94	9.1	12.8	0.71
	Pre	27.0	27.9	0.97				1.9	1.4	1.36
	19	12.6	20.4	0.62	7.6	0.8	9.5	6.8	6.9	0.99
	123	16.4	19.8	0.83	2.7	1.8	1.5	2.3	3.6	0.64
	166	21.4	24.1	0.88	2.23	3.8	0.59	10.9	10.3	1.06
LG	Pre	38.1	37.8	1.01	39.4	34.3	1.15	7.8	8.0	0.98
	Pre	34.2	33.4	1.02	13.0	14.9	0.87	4.9	4.7	1.04
	21	1.6	41.5	0.04	4.3	13.7	0.31	2.3	24.6	0.09
	56	4.4	16.1	0.27	2.1	7.3	0.29	2.6	10.8	0.24
AN	Pre	41.8	42.3	0.99	2.2	1.3	1.69	13.4	13.1	1.02
	Pre	36.7	36.6	1.00	4.7	4.3	1.09	8.0	9.9	0.81
	8	10.1	42.8	0.24	1.8	2.1	0.86	6.2	3.1	2.0
	22	25.5	25.9	0.98	2.3	7.2	0.32	20	21.9	0.91

* TK = Transplanted kidney.
† IK = Intact kidney.
‡ Pre = Pretransplantation studies.

TABLE IV

Glomerular filtration, sodium and potassium excretion rates in transplanted and intact kidneys *

Dog	Day following transplant	C_{Cr}† TK†	C_{Cr}† IK†	C_{Cr} TK/IK	$U_{Na}V$† *μEq./min.* TK	$U_{Na}V$† *μEq./min.* IK	$U_{Na}V$ TK/IK	Na Per cent of filtered load excreted TK	Na Per cent of filtered load excreted IK	U_KV *μEq./min.* TK	U_KV *μEq./min.* IK	U_KV TK/IK
					A. During infusion of 3% NaCl at 5.0 ml./min.							
PW	21	21.5	14.0	1.54	221	186	1.19	7.0	8.6	36	23.1	1.56
AN	21	39.8	35.3	1.13	86.8	295	0.29	1.4	5.5	16.5	29.8	0.55
LG	56	7.7	26.4	0.29	127	482	0.26	10.2	11.4	11.3	36.6	0.31
SP	125	29.1	40.9	0.71	618	883	0.70	14.3	14.0	32	47.2	0.68
					B. During infusion of isotonic solution at 8.8 ml./min.							
AN	8	9.1	47.7	0.19	5.2	101.6	0.05	0.49	1.51	3.2	7.2	0.44
SP	19	18.2	32.7	0.56	125.0	23.7	5.27	7.16	1.05	18.5	14.4	1.28
RQ	20	24.6	51.9	0.47	24.3	6.6	3.68	0.79	0.10	14.2	15.3	0.93
SP	123	23.9	33.2	0.72	165.4	280.3	0.59	5.04	6.13	10.7	12.8	0.84

* Data are mean values for three to nine clearance periods and do not include control periods. Solution used for isotonic expansion was a simulated ECF, containing Na, Cl, K, HCO_3, and glucose. Urinary losses of electrolytes and water were replaced during the experiment (see text).

† See footnotes to previous tables for meanings of these abbreviations.

these experiments revealed differences between the two kidneys, but there was no consistent abnormality noted in the patterns of the transplanted kidneys.

In Table IV-B results are shown for four experiments on three dogs. In two studies the percentage of filtered sodium excreted was greater for the transplanted kidney and in two slight to marked superiority of sodium excretion was exhibited by the intact kidney.

Wesson, Anslow, Raisz, Bolomey, and Ladd (6) have noted that if one selects successive points during isotonic expansion where the GFR and plasma sodium concentrations are identical (hence

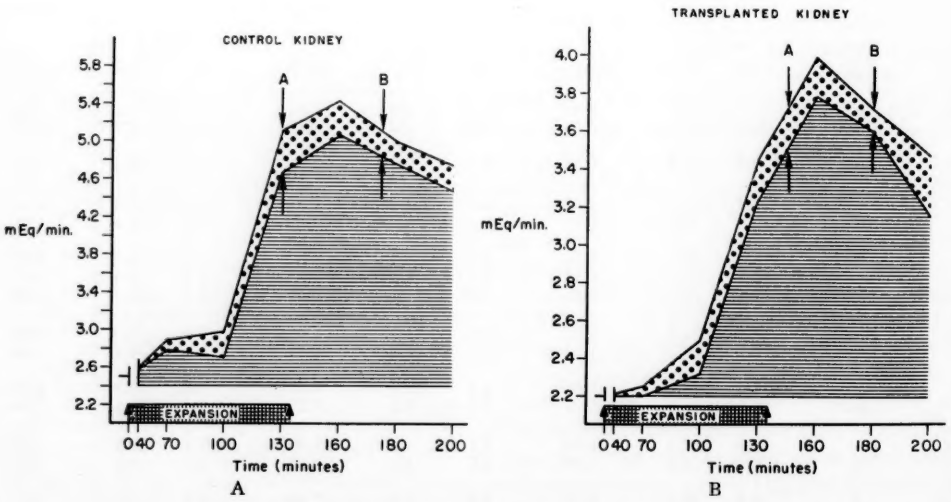

FIG. 4. NA REABSORPTION DURING ISOTONIC EXPANSION OF ECF

Entire area under outer curve represents filtered load of sodium; stipled area, Na excreted; cross-hatched area, Na reabsorbed. Time refers to minutes following onset of expansion (see text for details). Values for the three control periods are not included.

TABLE V

Summary of experiments on dogs with one kidney denervated by conventional means *

Dog	Experiment	Day following denervation	C_{Cr} *ml./min.* DK†	C_{Cr} *ml./min.* IK†	C_{Cr} DK/TK	$U_{Na}V$ *μEq./min.* DK	$U_{Na}V$ *μEq./min.* IK	Per cent FL_{Na} excreted DK	Per cent FL_{Na} excreted IK	Per cent FL_{Na} excreted DK/IK	U_KV *μEq./min.* DK	U_KV *μEq./min.* IK	U_KV DK/IK
HA	Isotonic expansion	Pre	43.3	43.4	1.0	308	232	5.2	4.1	1.27	55	47.4	1.16
	Isotonic expansion	11	25.9	45.9	0.56	147	212	3.8	3.2	1.19	22.1	31.5	0.70
	I.V. thiomerin	29	8.3	16.4	0.51	95	147	7.6	6.7	1.14	15.8	16.2	0.98
	3% NaCl	52											
	(a) before anes.		29.3	53.6	0.53	184	336	4.2	4.3	0.98	18.0	36.4	0.49
	(b) after anes.		25.6	50.2	0.51	348	530	8.4	7.3	1.15	18.6	32.3	0.58
VA	Isotonic expansion	Pre	37.4	40.7	0.92	222	206	3.5	3.3	1.06	20.1	19.8	1.01
	I.V. thiomerin	7	32.4	34.6	0.94	344	385	8.0	8.5	0.94	32.3	32.6	0.99
	Isotonic expansion	12	52.9	48.5	1.09	242	273	3.0	3.9	0.77	15.5	15.6	0.99
	3% NaCl	21											
	(a) before anes.		35.0	33.8	1.04	170	213	3.4	4.5	0.76	20.8	25.2	0.83
	(b) after anes.		41.0	32.1	1.32	448	246	7.4	5.2	1.42	20.8	11.2	1.86

* One predenervation experiment is included for each animal. Data are mean values for three to seven clearance periods and do not include control periods.

† DK refers to denervated kidneys, IK to intact kidneys. For all other abbreviations see footnotes to previous tables.

filtered loads are identical), the absolute rate of sodium reabsorption increases with time. On the basis of this observation, it has been suggested that the intrinsic reabsorptive capacity of the tubules increases in the dog as a function of volume expansion. In Figure 4, the results of the second experiment on Dog SP (Table IV-B) are depicted in the manner of Wesson and his colleagues for both the transplanted and intact kidneys. Comparing the rate of reabsorption at point B with point A in each diagram suggests that sodium reabsorption increased with time not only in the intact kidney but also in the transplanted kidney.

II. *Denervated nontransplanted kidneys*

Eleven clearance studies were performed on two dogs denervated by conventional means. The results of one control and three postdenervation experiments on each animal are shown in Table V. In one of the dogs, the GFR of the denervated kidney decreased from its preoperative level;[5] in the other, values for the two kidneys remained comparable after denervation. The results of these experiments do not differ in any major respect from those on the dogs in which one kidney was autotransplanted.

[5] This may have been a consequence of the fact that alcohol was injected into the adventitia of the renal artery to insure denervation.

DISCUSSION

The present studies were undertaken with a twofold purpose: *1*) to further define the functional capabilities of the transplanted kidney, and *2*) to examine the relationship between renal nerves and sodium excretion.

Experiments were performed on dogs with permanent hemibladders in which one kidney was autotransplanted and the opposite kidney left intact. Several properties of this preparation support its usefulness in such an investigation: *1*) construction of two separate urinary bladders provides the opportunity to study the functions of both kidneys in a single animal simultaneously and serially; *2*) autotransplantation of one kidney only permits comparison of the data of the transplanted organ both with its own pretransplantation values and with the concurrent responses of the intact organ; *3*) the removal of a kidney from the body of the host during the transplantation procedure results in the severing of all renal nerves without interrupting nerves to extrarenal splanchnic structures.

The results obtained serve to reinforce the impression gained from human observations (3) that a kidney, successfully transplanted, may function in most respects in a normal manner. The one major abnormality noted in the function of the autotransplanted kidney in the dog was a decrease

in glomerular filtration rate and renal plasma flow in the initial studies following transplantation. This defect did not persist, however, and in two animals the value for the GFR of the transplanted kidney ultimately came to equal that of the intact kidney. It seems probable that the initial decrease in clearance values resulted either from the period of anoxia sustained by the kidney during transplantation or from a temporary decrease in arterial or venous lumina at the anastamotic sites.

In recent years, interest has been reawakened in the possible influence of renal nerves on the tubular transport of sodium. In studies on dogs with one kidney denervated and the opposite one intact, the denervated organ has frequently been noted to excrete more sodium than the intact kidney. On the basis of the higher rates of sodium excretion by the denervated kidney, it has been suggested that renal nerves exert a specific effect on the renal tubular reabsorptive capacity for sodium chloride (8). In the majority of instances where differences in sodium excretion rates have existed, however, the denervated kidney has had a slightly greater filtration rate than the contralateral organ. It has been contended by several groups that the differences in the GFR (and thus the filtered loads of sodium) have been of sufficient magnitude to account for the differences in sodium excretion (10, 11). To date this conflict in interpretation has not been resolved.

In the majority of experiments performed in the present studies, filtration rate of the denervated kidney was *less* than the simultaneous value of the intact organ. A higher rate of sodium excretion by the transplanted kidney would, therefore, provide strong evidence in favor of a defect in tubular sodium reabsorptive capacity. Such evidence was not found. When sodium excretion rates were factored by their respective filtered loads, the ratios were not consistently greater for the transplanted kidneys and in several experiments they were less.

Expansion of extracellular fluid volume by infusion of 3 per cent sodium chloride resulted in the elaboration of a sodium-rich urine by both kidneys. In the unanesthetized state there was no evidence for impaired tubular reabsorption by the transplanted kidney. When anesthesia was induced the differences in sodium excretion between the two kidneys were explicable on the basis of concurrent changes in the respective glomerular filtration rates. When the GFR of the intact kidney decreased more than that of the transplanted kidney, superiority of sodium excretion (either relative to the filtered load or absolute) was exhibited by the transplanted kidney (Figure 2). Conversely, when the GFR of the transplanted kidney decreased more than that of the intact kidney (Table II), the opposite response was noted.

When the plasma sodium concentration was maintained constant and the filtered load of sodium rose due to increasing filtration rates (during isotonic expansion of ECF), the opportunity existed to investigate the effects of GFR on sodium excretion in transplanted and intact kidneys. Although differences in sodium excretion between the two kidneys were noted, these were not consistent in direction and no abnormal pattern for the transplanted kidney became apparent. In a dog studied five months after autotransplantation, expansion of ECF volume appeared to enhance tubular reabsorptive capacity for sodium not only in the intact kidney but also in the transplanted kidney.

The response of kidneys denervated by a conventional procedure (as described above) did not differ materially from that of kidneys denervated by autotransplantation. It, therefore, seems reasonable to conclude that denervation by conventional means provides satisfactory experimental models.

One theoretic objection to long-term studies on kidneys denervated by all procedures including transplantation is the possibility that nerve fibers may regenerate. Regeneration of nerve fibers into nerve tracts in the transplanted kidney has not yet been systematically investigated. Should this occur to any significant degree, it would not be expected until after the original nerve fibers had degenerated. In two-thirds of the present studies, data were obtained within three weeks of transplantation, and hence this objection would not apply. In the remaining studies, it cannot be asserted that regenerating fibers did not enter the kidney. Whether fibers from the iliac vessels (the sites of vessel anastomoses) could be expected to provide "normal" innervation for a kidney is beyond the scope of this discussion. However, the results of the more chronic experiments did not

differ consistently from the larger number of earlier studies.

SUMMARY AND CONCLUSIONS

Separate but simultaneous studies have been performed on the two kidneys of six dogs having one kidney autotransplanted into the iliac fossa and the opposite kidney intact. Data have been examined to obtain further information about *a*) the functional capacity of the transplanted kidney and, *b*) the effects of denervation (imposed by the transplantation procedure) on the tubular transport of sodium chloride. Serial studies were also performed on two dogs in which unilateral denervation was accomplished by a conventional procedure.

The results suggest *1*) the autotransplanted kidney in the dog demonstrated no persistent defect in any of the functional parameters examined, *2*) complete denervation occurring during the transplantation procedure did not result in any consistent abnormality in the tubular transport of sodium chloride, and *3*) the responses of the conventionally denervated kidneys did not differ in any major respect from the autotransplanted kidneys. Conventional denervation as employed in numerous previous studies, therefore, appears to be a satisfactory means of depriving the kidney of its nerve supply.

The possibility of nerve regeneration in the transplanted kidney has not yet been definitively studied. However, no consistent differences were noted between the long-term studies and those performed within three weeks of transplantation.

ACKNOWLEDGMENTS

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REFERENCES

1. Quinby, W. C. The function of the kidney when deprived of its nerves. J. exp. Med. 1916, **23**, 535.
2. Smith, H. W. The Kidney, Structure and Function in Health and Disease. New York, Oxford University Press, 1951.
3. Bricker, N. S., Guild, W. R., Reardan, J. B., and Merrill, J. P. Studies on the functional capacity of a denervated homotransplanted kidney in an identical twin with parallel observations in the donor. J. clin. Invest. 1956, **35**, 1364.
4. Desautels, R. Personal communication.
5. Murray, J. E., Lang, S., Miller, B. F., and Dammin, G. J. Prolonged functional survival of renal autotransplants in the dog. Surg. Gyn. Obstet. 1956, **103**, 15.
6. Wesson, L. G., Jr., Anslow, W. P., Jr., Raisz, L. G., Bolomey, A. A., and Ladd, M. Effect of sustained expansion of extracellular fluid volume upon filtration rate, renal plasma flow and electrolyte and water excretion in the dog. Amer. J. Physiol. 1950, **162**, 677.
7. Wesson, L. G., Jr. Glomerular and tubular factors in the renal excretion of sodium chloride. Medicine 1957, **36**, 281.
8. Kaplan, S. A., and Rapoport, S. Urinary excretion of sodium and chloride after splanchnicotomy; effect on the proximal tubule. Amer. J. Physiol. 1951, **164**, 175.
9. Kriss, J. P., Futcher, P. H., and Goldman, M. L. Unilateral adrenalectomy, unilateral splanchnic nerve resection and homolateral renal function. Amer. J. Physiol. 1948, **154**, 229.
10. Berne, R. M. Hemodynamics and sodium excretion of denervated kidney in anesthetized and unanesthetized dog. Amer. J. Physiol. 1952, **171**, 148.
11. Page, L. B., Baxter, C. F., Reem, G. H., Scott-Baker, J. C., and Smith, H. W. Effect of unilateral splanchnic nerve resection on the renal excretion of sodium. Amer. J. Physiol. 1954, **177**, 194.

STUDIES OF PULMONARY HYPERTENSION. IX. THE EFFECTS OF INTRAVENOUS HEXAMETHONIUM ON PULMONARY CIRCULATION IN PATIENTS WITH MITRAL STENOSIS [1]

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During the past five years, circulatory effects of intravenous hexamethonium have been studied in normal subjects and in patients with various cardiac diseases (1–10). With adequate doses of the drug a significant reduction of the systemic blood pressure was observed in almost all cases. In many patients, particularly those with pulmonary hypertension, a reduction of the pulmonary artery pressure was also seen. However, the pulmonary "capillary" or pulmonary artery wedge pressures were recorded in only a small number of cases and changes of resistances in the pulmonary circuit were seldom studied.

The purposes of this paper are: *1*) to report the hemodynamic effects of hexamethonium in a series of patients with mitral stenosis, *2*) to discuss the probable mechanism of the changes of the resistances and pressures in the pulmonary circuit of these patients, and *3*) to stress the therapeutic benefit of hexamethonium in patients with acute pulmonary edema secondary to mitral stenosis.

CLINICAL MATERIAL AND METHOD

Twenty-seven patients with predominant mitral stenosis were studied. There were 10 males and 17 females. Their ages ranged from 13 to 58 years. None of the patients had a significant degree of mitral insufficiency, a demonstrable lesion of other valves, or systemic hypertension.

The patients were studied two to three hours after a light breakfast. In all cases 100 to 200 mg. of methyprylon (Noludar®) [5] was given by mouth as premedication.

The methods of determining the cardiac output and recording blood pressures have been described in detail in several previous papers (11–14). The formulae used to derive the resistances and ventricular work against pressures and mitral valve flow were adopted from the papers by Gorlin and his co-workers (15, 16). The mean pressures were measured by planimetric integration. Pulmonary "capillary" pressure was substituted for pulmonary venous and left atrial pressures. Pulmonary vascular resistance is the resistance between pulmonary artery and pulmonary vein. Total pulmonary resistance is the resistance between pulmonary artery and a hypothetical sink at atmospheric pressure. The difference between total pulmonary resistance and pulmonary vascular resistance is termed "left heart resistance" instead of "mitral valve resistance" (17, 18). It includes the resistance at the mitral valve as well as the resistance to filling offered by the left ventricle in diastole. In patients with pure mitral stenosis, however, most of the calculated value is ascribable to resistance at the valve itself.

The right ventricular diastolic pressure was used in calculating the right ventricular work against pressure. In most cases right ventricular diastolic pressure was only recorded after hexamethonium administration and this was used for calculation of the work both before and after the drug. In several cases the control right ventricular diastolic pressure was little different from the value after injection. It was felt that the error would be small in calculating right ventricular work against pressure by using only the value following injection.

In calculating left ventricular work against pressure, the left ventricular diastolic pressure was assumed to be 5 mm. Hg in each case, because no left ventricular pressure was obtained at the time of cardiac catheterization. The calculation was discarded in five patients whose left ventricular diastolic pressure was later found, at operation, to exceed 8 mm. Hg.

After control measurements of cardiac output and femoral and pulmonary artery pressures (in all patients) and of pulmonary "capillary" pressure (in all but three

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[5] Courtesy of Hoffman LaRoche Company, Nutley, N. J.

TABLE I

Hemodynamic data in patients with mitral stenosis before and after intravenous injection of hexamethonium *

Case No.	Age	Sex	BSA	Dosage of C_6 (*mg.*)	Periods of study	R.Q.	Blood flow: O_2 cons.	A-V O_2 diff.	C.I.	H.R.	S.I.	O_2 saturation (%)
1. J. K.	34	M	2.00	12	I	0.86	144	41.5	3.47	90	39	92.9
					II (25)	0.85	143	41.4	3.46	100	34	92.7
2. R. B.	55	F	1.57	5	I	0.82	157	39.6	3.96	125	32	90.2
					II (16)	0.75	134	37.8	3.54	108	33	89.6
3. C. W.	56	M	2.10	6	I	0.87	165	55.4	2.97	80	37	96.1
					II (22)	0.92	146	56.0	2.61	82	32	98.3
					(45)	0.87	164	52.5	3.12	88	35	95.8
4. J. P.	36	F	1.63	10	I	0.80	109	51.1	2.13	67	32	94.1
					II (33)	0.78	128	51.1	2.50	70	36	96.2
					(80)	0.79	104	50.6	2.05	48	43	91.6
5. G. W.	30	F	1.60	6	I	0.84	128	38.0	3.37	118	29	99.6
					II (31)	0.78	132	38.9	3.39	126	27	92.3
6. A. W.	48	M	1.90	9	I	0.87	128	48.6	2.64	81	33	90.6
					II (32)	0.82	131	44.5	2.95	90	33	91.7
7. D. C.	13	F	1.39	6	I	0.89	134	44.3	3.02	103	29	98.0
					II (29)	0.88	144	42.2	3.41	106	32	95.0
8. M. B.	17	M	1.90	7	I		159	51.2	3.10	93	34	98.0
					II (25)			50.6		106		95.0
9. S. M.	40	F	1.51	10	I	0.80	129	53.2	2.40	90	27	94.3
					II (23)	0.88	131	53.8	2.40	100	24	95.0
					(41)	0.90	125	53.5	2.30	75	31	93.7
10. J. S.	26	M	1.64	9	I	0.83	151	55.4	2.72	92	30	91.6
					II (25)	0.85	138	49.8	2.79	88	32	94.6
					(44)	0.74	147	45.6	3.22	83	39	92.6
11. N. M.	41	M	1.78	9	I	0.83	150	62.7	2.39	80	30	96.2
					II (31)	0.85	134	66.5	2.02	100	20	96.1
					(73)	0.80	130	66.1	1.97	90	22	95.8
12. G. P.	38	F	1.51	14	I		112	52.6	2.15	88	24	99.5
					II (45)		107	53.6	2.00	86	23	94.1
13. M. S.	24	F	1.40	2	I	0.81	132	64.6	2.05	95	22	96.4
					II (29)	0.83	138	65.9	2.09	108	19	94.3
14. M. F.	58	F	1.61	8	I	0.77	137	74.3	1.85	69	27	94.2
					II (24)	0.85	118	53.9	2.19	74	30	90.2
					(49)	0.89	124	57.3	2.16	63	34	90.4
15. K. K.	37	F	1.67	22.5	I		144	59.0	2.44	90	27	91.7
					II (27)		125	54.1	2.30	90	26	89.2
					(72)		121	56.0	2.16	84	26	90.5
16. A. F.	47	M	1.66	8	I	0.81	125	72.0	1.70	73	24	90.0
					II (23)	0.83	125	74.7	1.70	97	17	89.0
					(72)	0.80	135	77.1	1.80	87	20	89.0
17. E. W.	30	F	1.55	18	I		131	68.1	1.93	94	21	91.1
					II (34)		145	60.0	2.42	94	26	88.4
18. A. K.	36	F	1.40	6	I	0.85	128	57.0	2.24	88	25	96.4
					II (15)	0.85	136	53.1	2.56	88	29	92.7
					(42)	0.90	128	58.7	2.18	71	30	90.8
19. R. K.	53	M	1.80	15	I	0.88	116	75.7	1.53	66	23	99.3
					II (30)	0.90	112	60.6	1.85	70	26	95.9
20. H. J.	41	F	1.48	14	I	0.84	158	73.4	2.15	80	27	92.9
					II (50)	0.91	131	50.0	2.62	69	38	90.2
21. E. J.	47	F	1.44	7	I	0.95	113	77.4	1.46	92	16	99.7
					II (28)	0.90	96	58.2	1.65	88	19	93.3
					(64)	0.85	112	62.7	1.78	78	23	93.0
22. B. K.	34	F	1.72	6	I		118	99.1	1.19	102	12	89.8
					II (30)		94	79.6	1.19	78	15	84.4
23. A. C.	47	M	1.86	10	I	0.88	164	65.7	2.45	74	33	91.3
					II (47)	0.91	149	54.5	2.16	84	26	90.6
24. M. Mc.	55	F	1.49	14	I	1.03	115	52.6	2.19	96	23	97.0
					II (39)	0.77	105	60.6	1.74	107	16	98.0
25. G. A.	39	F	1.51	11	I		129	59.7	2.16	82	27	99.2
					II (29)		113	55.5	2.04	81	25	95.2
26. D. R.	42	M	1.86	9	I	0.96	109	37.4	3.34	84	40	91.8
					II (29)	0.97	110	55.3	2.16	80	27	94.0
					(48)		113	48.6	2.35	85	28	92.0
27. J. Sc.	40	F	1.66	12	I	0.88	120	51.6	2.33	98	23	90.9
					II (35)	0.80	144	44.7	3.22	98	32	90.9

*BSA = Body surface area ($M.^2$).
C_6 = Hexamethonium ion.
I = Control period.
II = Period following C_6 injection; the figures in the parentheses indicate the minute after the start of injection of C_6.
R.Q. = Respiratory quotient.

O_2 cons. = Oxygen consumption (ml./$M.^2$/min., STPD)
A-V O_2 diff. = Arteriovenous oxygen difference (ml./L.).
C.I. = Cardiac index (L./$M.^2$/min.).
H.R. = Heart rate (beats/min.).
S.I. = Stroke index (ml./$M.^2$/beat).

TABLE I—*Continued*

Case No.	Periods of study	Pressures (mm. Hg) F.A. (S/D)	F.A. (M)	P.A. (S/D)	P.A. (M)	"P.C." (M)	P.A.–"P.C." (M)	R.V. (S/D)	R.A. (M)	L.V. (D)	Resistances (dynes sec./cm.^{5}) TSR	TPR	PVR	"LHR"	Ventricular work against pressure Left (Kg.M./M.^{2})	Right
1. J. K.	I	102/63	74	50/28	36	29	7			14	830	410	80	330		1.5
	II (25)	95/59	72	46/24	34	28	6	62/6	5		840	380	60	320		1.4
2. R. B.	I	166/74	104	47/29	35	29	6			10	1,320	440	80	360		1.8
	II (16)	127/66	87	34/21	24	16	8	43/3	1		1,230	340	110	220		1.1
3. C. W.	I	180/90	118	73/30	43	28	15	70/7		6	1,490	540	190	350	4.8	1.5
	II (22)	103/49	65	36/19	25	13	12				930	360	170	190	2.2	0.7
	(45)	109/64	81	44/26	32			44/5	5		970	390			3.3	1.2
4. J. P.	I	102/52	71	40/18	25	18	7			6	1,610	570	160	410	2.0	0.6
	II (33)	83/36	58	26/15	19						1,120	370			1.9	0.5
	(80)	86/38	48	28/12	16			30/6	4		1,110	380			1.3	0.3
5. G. W.	I	104/73	87	66/40	47	37	10			8	1,130	640	240	400	4.0	2.1
	II (31)			59/36	41	27	14	57/4	4		1,100	610	210	400		1.8
6. A. W.	I	148/76	102	65/34	46	38	8			13	1,620	730	220	510		1.4
	II (32)	124/76	93	62/32	42	29	13	65/11	10		1,330	600	190	410		1.3
7. D. C.	I	92/60	72	60/30	42	29	13			1	1,350	790	240	550	2.9	1.3
	II (29)	96/60	75	55/35	43	36	7	55/5	5		1,250	710	120	590	3.4	1.8
8. M. B.	I	105/61	74	80/46	60	30	30	75/8	7		1,010	820	410	410	3.0	2.3
	II (25)	84/60	66	67/40	49	30	19	67/9	7							
9. S. M.	I	120/71	89	66/29	41	26	15			0	1,910	880	320	560	2.9	1.3
	II (23)	87/50	63	45/23	32	16	16				1,350	690	340	340	2.0	1.0
	(41)	99/55	71	37/14	25	11	14	37/4	2		1,630	570	320	250	2.0	0.8
10. J. S.	I	133/83	100	80/37	55	33	22			6	1,760	950	390	560	3.7	2.0
	II (25)	100/60	76	69/35	47	30	17				1,310	810	290	520	2.8	1.7
	(44)	110/61	77	56/27	38	20	18	50/3	2		1,150	570	270	300	3.3	1.6
11. N. M.	I	116/62	80	87/38	51	25	26			7	1,670	960	490	470	2.6	1.6
	II (31)	87/53	65	66/39	48						1,440	1,070			1.8	1.2
	(73)	92/59	68	80/38	50			67/7	7		1,550	1,140			1.8	1.2
12. G. P.	I	148/87	102	52/30	36	24	12			3	2,470	1,010	320	690	3.0	1.0
	II (45)	160/91	115	40/25	30	18	13	43/3	5		3,000	810	340	470	3.1	0.7
13. M. S.	I	119/76	90	61/34	46			61/9	7	9	2,560	1,280				1.1
	II (29)	98/61	72	57/32	45			52/7	6		2,150	1,230				1.2
14. M. F.	I	163/85	114	80/28	44	21	23			16	3,000	1,310	750	560		0.9
	II (24)	95/52	63	72/28	43	17	26				1,430	970	590	380		1.0
	(49)	104/53	69	70/25	40	18	22	68/11	10		1,580	920	500	420		0.9
15. K. K.	I	110/74	91	127/43	73	36	37			2	1,780	1,430	720	710	3.0	2.4
	II (27)	103/62	76	104/38	59	26	33				1,570	1,210	680	530	2.3	1.8
	(72)	96/58	72	94/36	56	22	34	81/4	4		1,590	1,240	750	490	2.1	1.6
16. A. F.	I	120/57	81	80/36	53	31	22	91/6		6	2,250	1,470	610	860	1.8	1.2
	II (23)	82/52	63	70/38	49	23	26				1,820	1,420	750	670	1.4	1.0
	(72)	98/54	70	82/42	56	26	30	78/5	7		1,900	1,520	820	700	1.7	1.3
17. E. W.	I	104/60	76	86/42	56	24	32			2	2,020	1,490	850	640	2.0	1.4
	II (34)	85/52	62	70/38	50	23	27	70/7	5		1,320	1,060	570	490	2.0	1.6
18. A. K.	I	123/72	90	105/38	61	35	26			5	2,370	1,550	660	890	2.7	1.8
	II (15)	85/46	60	86/38	55	24	31				1,340	1,230	680	550	2.0	1.8
	(42)	85/44	55	69/33	44	18	26	50/7	6		1,440	1,150	670	480	1.6	1.2
19. R. K.	I	118/64	82	100/36	56	25	29			6	2,370	1,620	900	720	1.7	1.0
	II (30)	98/54	65	78/33	47			67/7	9		1,570	1,140			1.6	1.0
20. H. J.	I	158/84	100	113/45	66	30	36				2,560	1,690	920	770	2.9	1.8
	II (50)	141/72	85	78/34	44	21	23	77/9	9		1,740	900	430	470	3.0	1.3
21. E. J.	I	121/79	98	80/44	55	30	25	79/9	10		3,650	2,160	940	1,220	1.9	1.0
	II (28)	78/51	60	66/30	40	22	18				2,000	1,320	600	720	1.3	0.7
	(64)	94/54	66	68/30	41	21	20	63/6	7		2,060	1,260	620	640	1.6	0.9
22. B. K.	I	108/74	87	126/61	86					4	3,370	3,320			1.4	1.3
	II (30)	89/59	68	103/41	61			95/11	10		2,640	2,360			1.1	0.9
23. A. C.	I	127/74	88	84/33	47			72/5		2	1,500	800			2.9	1.5
	II (47)	74/43	53	49/25	35			49/4	3		1,040	690			1.6	1.0
24. M. Mc.	I	150/75	106	38/19	26	18	8			4	2,620	640	190	450	3.1	0.7
	II (39)	138/74	94	37/18	25	14	11	37/3	2		3,350	900	470	430	2.2	0.5
25. G. A.	I	166/81	114	61/31	42	27	15			1	2,750	1,000	360	640	3.1	1.6
	II (29)	108/55	75	42/23	29	13	16	41/3	2		1,920	740	410	330	2.0	0.8
26. D. R.	I	122/70	95	50/28	35	22	13			5	1,230	450	170	280	4.3	1.4
	II (29)			36/17	23	14	9					460	180	280		0.6
	(48)	88/50	63	37/16	23	14	9	35/4	6		1,170	430	170	260	1.9	0.6
27. J. Sc.	I	114/81	92	86/44	63	36	27		7		1,900	1,250	510	740	2.9	1.8
	II (35)	94/54	69	82/44	60			82/3	3		1,010	880			2.9	2.6

F.A. = Systemic artery pressure.
P.A. = Pulmonary artery pressure.
"P.C." = Pulmonary "capillary" or pulmonary artery wedge pressure.
P.A.–"P.C." = Pulmonary artery to pulmonary "capillary" pressure gradient.
R.V. = Right ventricular pressure.
R.A. = Right atrial pressure.
L.V. = Left ventricular pressure (obtained at operation).
S = Systolic; D = Diastolic; M = Mean.
TSR = Total systemic resistance.
TPR = Total pulmonary resistance.
PVR = Pulmonary vascular resistance.
"LHR" = "Left heart resistance."

patients), hexamethonium bromide (Hexameton®) [6] diluted in physiological saline was injected through the catheter at a rate of about 1 mg. of ion per minute. Injection was stopped when the brachial artery systolic pressure, measured at one minute intervals with a sphygmomanometer, had fallen 30 to 40 mm. Hg. The total dose of ion was 2 mg. in 1 case, between 6 and 10 mg. in 17 cases, and 11 mg. or more in the remaining 9 cases. In most cases the systemic blood pressure stabilized at the reduced level during the following 30 to 60 minutes. In a few patients there was a further drop in blood pressure one hour or more after the start of the injection. In two patients (D.C. and G.P.), the systemic blood pressure returned to the control level before the cardiac output determinations were repeated.

In all but one patient (M.B.), one or more determinations of cardiac output were made following hexamethonium injection, after which pulmonary artery and femoral artery pressures were again recorded in each case. Pulmonary "capillary" pressures were obtained in 20 cases. The respective data obtained were used for comparison and statistical analysis.

In any hemodynamic study there are limits of variation for each determinant. These have been studied in our laboratory and some of the findings reported previously (14, 18). In addition, studies were made on a series of 20 patients with two successive control determinations of systemic artery pressure, total systemic resistance, left and right ventricular works against pressure. A summary of the ranges of variation of all the determinants for the repeated measurements in the same patient is given below.

Cardiac index (C.I.)	+13 per cent to −10 per cent
Stroke index (S.I.)	+7 ml. to −5 ml. per M.² per beat
Systemic artery mean pressure (FA_m)	±15 mm. Hg
Pulmonary artery mean pressure (PA_m)	+5 mm. Hg to −8 mm. Hg
Pulmonary "capillary" mean pressure ("PC"$_m$)	±5 mm. Hg
PA_m−"PC"$_m$ gradient (PA_m−"PC"$_m$)	±4 mm. Hg
Total systemic resistance (TSR)	±25 per cent
Total pulmonary resistance (TPR)	+20 per cent to −30 per cent
Pulmonary vascular resistance (PVR)	±25 per cent
"Left heart resistance" ("LHR")	±20 per cent
Left ventricular work against pressure (LVW)	±25 per cent
Right ventricular work against pressure (RVW)	±25 per cent

Deviations of individual results beyond these limits are referred to hereafter as "individually significant" in-

[6] Courtesy of Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y.

TABLE II

Statistical data in hemodynamic changes following hexamethonium injection *

	No. of cases	Control *mean*	Hexamethonium *mean*	Standard error of difference	Difference as per cent of control *mean*	p†
Blood flow						
C.I.	26	2.39	2.44	0.06	+ 2	>0.1
S.I.	26	27.4	28.2	1.32	+ 3	>0.1
H.R.	27	98	98	2.11	0	>0.1
Blood pressures						
FA_m	26	91.9	72.6	2.67	−21.0	<0.005
PA_m	27	46.9	37.7	1.15	−18.5	<0.005
"PC"$_m$	20	28.9	22.0	1.29	−24.1	<0.005
PA_m−"PC"$_m$	20	18.3	16.0	1.14	−12.6	0.05<p<0.1
Resistances						
TSR	26	2,042	1,594	9.32	−21.9	<0.005
TPR	26	1,128	868	5.52	−31.9	<0.005
PVR	19	434	370	4.03	−14.7	>0.1
"LHR"	19	596	419	3.43	−29.7	<0.005
Ventricular work against pressure						
LVW	20	2.78	2.19	0.152	−21.2	<0.005
RVW	26	1.42	1.19	0.072	−16.3	<0.005

* The abbreviations and units in this table are identical to those used in Table I.
† The symbol of "p" indicates the probability that a difference as large as that observed will occur by chance. Throughout the discussion, a difference with a chance probability of 0.05 or less is considered to be significant.

TABLE III

Individually significant differences in the hemodynamic determinants following hexamethonium injection *

	Total	No. of cases showing individually significant increase	No. of cases showing individually significant decrease
Blood flow			
C.I.	26	7	4
S.I.	25	4	4
Pressures			
FA_m	26	0	19
PA_m	25	0	14
"PC"$_m$	20	1	14
PA_m–"PC"$_m$	20	2	5
Resistances			
TSR	26	2	14
TPR	26	1	12
PVR	19	3	6
"LHR"	19	0	14
Ventricular work against pressure			
LVW	20	0	12
RVW	26	2	12

* The abbreviations and units in this table are identical to those used in Table I.

creases or decreases. The term "statistically significant" refers to differences between two means having a "p" value less than 0.05.

A few days or weeks following the cardiac catheterization, mitral valvuloplasty was performed by Dr. E. B. Mahoney on all but three patients (M.B., H.J., and J.Sc.). At operation, atrial and left ventricular pressures were recorded immediately before and after fracture of the mitral valve in 23 cases. A pressure gradient of 6 mm. Hg or more across the mitral valve during diastole was found in all but one patient (R.B.). The left ventricular diastolic pressure was 8 mm. Hg or less in 18 of the 23 cases. In the remaining five cases, the left ventricular diastolic pressures were 9, 12, 13, 14 and 16 mm. Hg and suggested the presence of concomitant left ventricular failure.

RESULTS

The results are given in detail in Table I and summarized in Tables II and III. It will be seen from Table II that following hexamethonium injection there were statistically significant decreases in femoral artery pressure, pulmonary artery pressure, and pulmonary "capillary" pressure, but no statistically significant changes in cardiac index, stroke index, heart rate, pulmonary artery to pulmonary "capillary" pressure gradient and pulmonary vascular resistance. The total systemic, total pulmonary, "left heart" resistances (Figure 1), and the work of each ventricle against pressure were all statistically significantly reduced. The incidence of individually significant changes in these determinants is given in Table III.

The decrease in pulmonary artery pressure usually occurred within 10 minutes of the start of the injection, and at the same time as, or shortly following, the fall in femoral artery pressure. The systolic pressure in the pulmonary artery was more affected than the diastolic. The fall in mean pres-

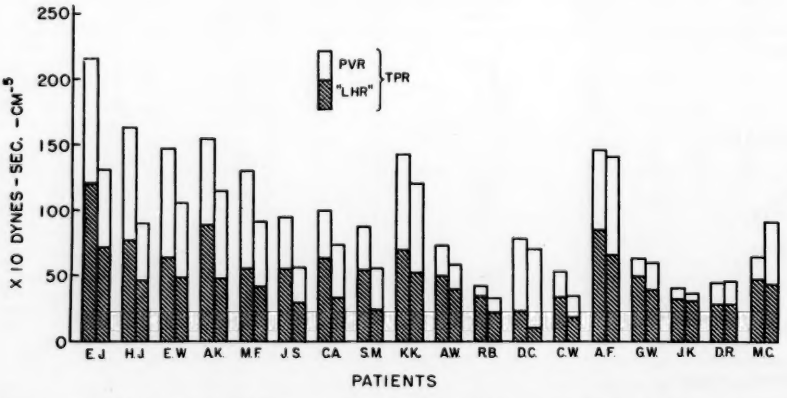

FIG. 1. CHANGE IN TOTAL PULMONARY, PULMONARY VASCULAR AND "LEFT HEART" RESISTANCES FOLLOWING HEXAMETHONIUM INJECTION IN 18 PATIENTS WITH MITRAL STENOSIS

The first column of each pair indicates the resistances measured during the control period and the second column shows the resistances after hexamethonium injection.

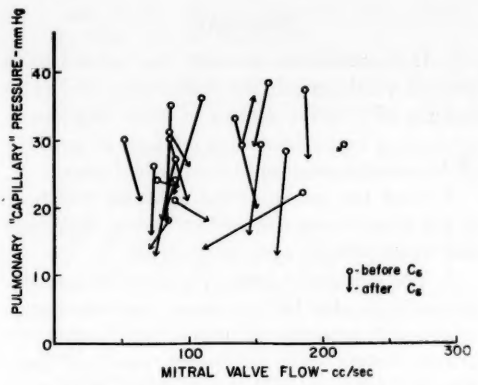

FIG. 2. RELATION BETWEEN PULMONARY "CAPILLARY" PRESSURE AND MITRAL VALVE FLOW BEFORE AND AFTER HEXAMETHONIUM

The pressure changes are unrelated to the trivial alterations in flow.

sure was, in most cases, proportionally similar in the pulmonary and femoral arteries.

The decrease in pulmonary "capillary" pressure was also roughly proportional, in most cases, to the decrease in the femoral artery pressure. The change in pulmonary "capillary" pressure was unrelated to changes in flow, whether the latter was expressed per minute, per heart beat, or per second of diastolic time (Figure 2). It was also unrelated to the change in pulmonary vascular resistance. The "capillary" pressure changes were significantly positively correlated with the changes in "left heart resistance," left ventricular work against pressure, and right ventricular work against pressure.

Use of intravenous hexamethonium in pulmonary edema

Intravenous hexamethonium was used clinically with excellent response in treating acute pulmonary edema in three patients with mitral stenosis. In two additional patients with mitral stenosis, acute pulmonary edema which developed during cardiac catheterization was promptly controlled after the injection of hexamethonium through the catheter. In the first case the pulmonary artery pressure rose from a control value of 124/65 (mean, 84) to 140/100 mm. Hg (mean, 113) during acute pulmonary edema. A total of 10 mg. of hexamethonium ion was given over a period of 15 minutes, during which time the pulmonary artery pressure decreased to 92/44 mm. Hg (mean, 58). In the other case, the pulmonary artery pressure was 72/38 mm. Hg (mean, 47) at rest, rose to 130/70 mm. Hg during acute pulmonary edema, and decreased to 51/27 mm. Hg (mean, 35) 20 minutes after injection of 12 mg. of hexamethonium ion. The details of these observations will be reported in a separate communication.

DISCUSSION

Our results are in agreement with several reports that ganglionic blocking agents, given to patients with mitral stenosis, reduce the pulmonary artery and pulmonary "capillary" pressures without significantly altering the flow (5, 9, 10, 19). This pattern is different from that commonly observed in normal animals (20, 21), in patients without cardiorespiratory disease (4), and in a variety of cardiorespiratory diseases other than mitral stenosis (1, 6, 8, 22). In these groups, cardiac output usually falls sufficiently to account for the decline in pulmonary and systemic pressures without invoking changes in resistance. This type of response, ascribed to peripheral pooling, was found by Storstein and Tveten (6) to occur also in eight patients with mitral stenosis. We have no explanation for the discrepancy between their results in this disease and those of others including ourselves.

The paradox of reduced pulmonary "capillary" pressure without change in flow has not, to our knowledge, been satisfactorily explained. The explanation cannot lie simply in vasomotor changes in any part of the lung, nor in passive shifts of blood to the periphery, for any change in left atrial pressure so induced would of necessity change the flow also.

There are two possible explanations which seem to us most likely to be correct: *1*) a drop in left ventricular diastolic pressure, and *2*) intrapulmonary shunting of blood.

Only five of our patients were found later to have abnormally high left ventricular diastolic pressures, and these were not those who had the most marked drops of pulmonary "capillary" pressure at catheterization.

Nevertheless, a decline of this pressure follow-

ing hexamethonium has been noted in normal dogs (19) and it would, of course, permit a similar drop to occur in the left atrial pressure without reducing the flow.

Intrapulmonary shunts connecting the pulmonary vein to the azygos vein and right atrium by way of varicose anastomoses with the bronchial vein are known to exist in mitral stenosis (23). When the pulmonary veins of one lung are ligated in dogs, such anastomoses may carry up to 20 per cent of the normal flow for that lung, so that azygos vein blood resembles arterial blood (24). If such anastomoses in mitral stenosis patients were subject to dilation by hexamethonium, as systemic veins appear to be, then the paradox could be explained, as would the difference in response between mitral stenosis and other diseases. The increased low-pressure runoff would probably lower pulmonary venous pressure, which in turn would reduce the flow through the mitral valve. The shunted arterialized blood would recirculate through the lungs. The change in pulmonary flow, as we measured it, might therefore be nil, or even an increase, depending on the balance between increase in shunt flow and decrease in mitral valve flow.

Among other explanations we have discarded an actual reduction of mitral valve resistance as highly unlikely on structural grounds. It is true that marked slowing of the heart rate was associated with the largest decrease in pulmonary "capillary" pressure per unit flow. However, the overall change in heart rate was insignificant, and even when the length of diastole is taken into account, there were no flow changes to correlate with the alterations in pressure (Figure 2). Lastly, it seems unlikely that we are dealing with vasomotor changes in the recently postulated zone of resistance in the pulmonary venous bed (25).

The decline in pulmonary vascular resistance, which was noted in six cases, it not likely to result passively from the lowering of left atrial pressure, since this should have the opposite effect (26). Neither does it seem likely to be due to a vasodilator reflex from pressure receptors in the pulmonary capillary-venous bed (9), since the changes were unrelated to changes in pulmonary "capillary" pressure. It is possible that it was caused by ganglionic blockade of the pulmonary arteriolar bed (2, 5, 19).

SUMMARY

1. Hexamethonium bromide was administered through a catheter into the pulmonary artery of 27 patients with mitral stenosis in doses sufficient to produce an initial depression of about 30 mm. Hg in the systolic pressure in the brachial artery.

2. Over the course of the following half-hour to one hour, changes in cardiac output, heart rate and stroke volume were insignificant.

3. The pulmonary artery pressure declined significantly in over half the cases, and the change was roughly proportional to the change in systemic artery pressure. The pulmonary "capillary" pressure also fell in more than two-thirds of observations. There was no consistent change in the PA_m–"$PC"_m$ gradient, although pulmonary vascular resistance fell in a few instances.

4. The paradox of declining pulmonary "capillary" pressure with constant cardiac output is discussed. On hydraulic grounds it is unlikely that this could be explained solely by any vasomotor changes in the lung vessels or by passive shifts of blood from pulmonary to peripheral reservoirs, although both these may occur in addition. Changes in heart rate may play a small contributing role. Two other possible explanations are discussed: changes in left ventricular diastolic pressure, and increases in the transport of blood through intrapulmonary vascular shunts.

5. The clinical importance of the action of hexamethonium on the pressure in pulmonary circuit is illustrated by two cases of its successful use in the treatment of acute pulmonary edema which developed in patients with mitral stenosis during cardiac catheterization.

ACKNOWLEDGMENT

We wish to express our thanks and appreciation to Dr. S. Lee Crump, Associate Professor of Radiation Biology and Scientist (Statistics), Atomic Energy Project, University of Rochester, for his help on the statistical aspects of this study. We are indebted to Mrs. Ruth Knight for technical assistance, and to Miss Irene Subrani for preparing the manuscript.

REFERENCES

1. Werkö, L., Frisk, A. R., Wade, G., and Eliasch, H. Effect of hexamethonium bromide in arterial hypertension. Lancet 1951, **2**, 470.
2. Gilmore, H. R., Kopelman, H., McMichael, J., and Milne, I. G. The effect of hexamethonium bro-

mide on the cardiac output and pulmonary circulation. Lancet 1952, **2**, 898.

3. Kelley, R. T., Freis, E. D., and Higgins, T. F. The effects of hexamethonium on certain manifestations of congestive heart failure. Circulation 1953, **7**, 169.
4. Rakita, L., and Sancetta, S. M. Acute hemodynamic effects of hexamethonium in normotensive man. Circ. Res. 1953, **1**, 499.
5. Davies, L. G., Goodwin, J. F., and VanLeuven, B. D. The nature of pulmonary hypertension in mitral stenosis. Brit. Heart J. 1954, **16**, 440.
6. Storstein, O., and Tveten, H. The effect of hexamethonium bromide on the pulmonary circulation. Scand. J. clin. Lab. Invest. 1954, **6**, 169.
7. Burch, R. R. The effects of intravenous hexamethonium on venous pressure of normotensive and hypertensive patients with and without congestive heart failure. Circulation 1955, **11**, 271.
8. Sancetta, S. M. Acute hemodynamic effects of hexamethonium (C_6) in patients with emphysematous pulmonary hypertension. Amer. Heart J. 1955, **49**, 501.
9. Wade, E. G., Mackinnon, J., and Vickers, C. F. H. The nature of the increased pulmonary vascular resistance in mitral stenosis. Brit. Heart J. 1956, **18**, 458.
10. Balchum, O. J., Gensini, G., and Blount, S. G., Jr. The effect of hexamethonium upon the pulmonary vascular resistance in mitral stenosis. J. Lab. clin. Med. 1957, **50**, 186.
11. Yu, P. N., Lovejoy, F. W., Jr., Joos, H. A., Nye, R. E., Jr., and McCann, W. S. Studies of pulmonary hypertension. I. Pulmonary circulatory dynamics in patients with pulmonary emphysema at rest. J. clin. Invest. 1953, **32**, 130.
12. Yu, P. N., Simpson, J. H., Lovejoy, F. W., Jr., Joos, H. A., and Nye, R. E., Jr. Studies of pulmonary hypertension. IV. Pulmonary circulatory dynamics in patients with mitral stenosis at rest. Amer. Heart J. 1954, **47**, 330.
13. Yu, P. N., Lovejoy, F. W., Jr., Joos, H. A., Nye, R. E., Jr., Beatty, D. C., and Simpson, J. H. Studies of pulmonary hypertension. VI. Pulmonary "capillary" pressure in various cardiopulmonary diseases at rest and under stress. Amer. Heart J. 1955, **49**, 31.
14. Yu, P. N., Beatty, D. C., Lovejoy, F. W., Jr., Nye, R. E., Jr., and Joos, H. A. Studies of pulmonary hypertension. VII. Hemodynamic effects of acute hypoxia in patients with mitral stenosis. Amer. Heart J. 1956, **52**, 683.
15. Gorlin, R., and Gorlin, S. G. Hydraulic formula for calculation of the area of the stenotic mitral valve, other cardiac valves, and central circulation shunts. Amer. Heart J. 1951, **41**, 1.
16. Gorlin, R., Haynes, F. W., Goodale, W. T., Sawyer, C. G., Dow, J. W., and Dexter, L. Studies of the circulatory dynamics in mitral stenonsis. II. Altered dynamics at rest. Amer. Heart. J. 1951, **41**, 30.
17. Werkö, L., Biörck, G., Crafoord, C., Wulff, H., Krook, H., and Eliasch, H. Pulmonary circulatory dynamics in mitral stenosis before and after commissurotomy. Amer. Heart J. 1953, **45**, 477.
18. Yu, P. N., Nye, R. E., Jr., Lovejoy, F. W., Jr., Macias, J. deJ., Schreiner, B. F., and Lux, J. J. Studies of pulmonary hypertension. VIII. The effects of acetyl strophanthidin on pulmonary circulation in patients with cardiac failure and mitral stenosis. Amer. Heart J. 1957, **54**, 235.
19. Scott, R. C., Kaplan, S., and Stiles, W. J. Observations on the effect of tetraethylammonium chloride on the pulmonary vascular resistance in mitral stenosis. Amer. Heart J. 1955, **50**, 720.
20. Wakim, K. G. Certain cardiovasculorenal effects of hexamethonium. Amer. Heart J. 1955, **50**, 435.
21. Combes, B., Preedy, J. R. K., Wheeler, H. O., Hays, R. M., and Bradley, S. E. The hemodynamic effects of hexamethonium bromide in the dog, with special reference to "splanchnic pooling." J. clin. Invest. 1957, **36**, 860.
22. Smith, J. R., and Hoobler, S. W. Acute and chronic cardiovascular effects of pentolinium in hypertensive patients. Circulation 1956, **14**, 1061.
23. Ferguson, F. C., Gobilak, R. E., and Deitrick, J. E. Varices of the bronchial veins as a source of hemoptysis in mitral stenosis. Amer. Heart J. 1944, **28**, 445.
24. Hurwitz, A., Calabresi, M., Cooke, R. W., and Liebow, A. A. An experimental study of the venous collateral circulation of the lung. II. Functional observations. J. thorac. Surg. 1954, **28**, 241.
25. Tosetti, R. Les zones de résistance vasulaire pulmonaire au cours du retrécissement mitral et du coeur pulmonaire chroniquedos emphysémateux; étude de leur localisation. Arch. Mal. Coeur, 1955, **48**, 346. (Abstract in Circulation 1956, **13**, 784.)
26. Borst, H. G., McGregor, M., Whittenberger, J. L., and Berglund, E. Influence of pulmonary arterial and left atrial pressures on pulmonary vascular resistance. Circ. Res. 1956, **4**, 393.

THE EFFECT OF EXERCISE IN THE SUPINE POSITION ON PULMONARY VASCULAR DYNAMICS IN PATIENTS WITH LEFT-TO-RIGHT SHUNTS

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The effect of exercise on pulmonary artery pressure, blood flow, and resistance has been studied extensively in normal subjects (1–5), and in patients with rheumatic heart disease (6, 7). However, only brief reports have appeared concerning the response of patients with intracardiac or great vessel shunts (8, 9). The dearth of published information probably is due in part to uncertainty as to the exact diagnosis in a sufficient number of cases. This paper concerns the changes in pulmonary and systemic blood flows, pressures, and resistances, and the associated variations in intracardiac or aortopulmonary shunts caused by leg exercise in the supine position in 30 patients with single congenital anomalies. In those patients with pulmonary hypertension the pulmonary blood flow was found to change only slightly during exercise irrespective of the magnitude of the left-to-right shunt, while pulmonary artery pressure and pulmonary resistance values were increased. In patients without pulmonary hypertension there was a more definite increase in pulmonary blood flow; but as in the preceding group, a decrease was observed in the magnitude of the left-to-right shunt. Complete data for accurate anatomic appraisal had been obtained in all instances, and operative confirmation of the diagnosis was available in the great majority.

METHODS AND MATERIALS

Procedure. The following procedure was adhered to, with minor variations. The patient received 0.5 grain (30 mg.) of codeine and 1.5 grains of secobarbital (Seconal®) immediately prior to the start of the procedure. He lay supine on a horizontally positioned fluoroscopy table. A No. 5 or 6 Cournand cardiac catheter (designated catheter 1) was introduced, usually by percutaneous needle puncture of a vein in the right antecubital fossa (10) and advanced to the heart. Oxygen saturations of blood samples obtained from various locations within the heart and great vessels were measured by a cuvette oximeter (11) to ascertain the oxygen saturation of the mixed venous blood and to determine the magnitude and location of the left-to-right shunt. The catheter tip was located in the main pulmonary artery, and a needle was inserted into a radial artery. Pressures in the systemic and pulmonary artery systems were recorded by means of strain-gauge manometers. It was desirable in a number of patients to obtain simultaneously samples of blood from the inferior vena cava as well as from the pulmonary artery. To make this possible a second catheter (designated catheter 2) was inserted and advanced directly into the inferior vena cava. The rate of oxygen consumption was then determined by collection of the expired air for five minutes in a large-volume, low-resistance plastic balloon. Analysis of the expired air was carried out immediately by the Haldane method. Midway during the period of collection of expired air, blood samples were obtained for analysis of the oxygen content by the method of Van Slyke and Neill (12) from the pulmonary artery and the radial artery, and from the inferior vena cava if a second catheter was being used. The oxygen capacity of hemoglobin was measured by the method of Sendroy (13), using the modification of Roughton, Darling, and Root (14). Immediately following the collection of the blood samples, a record of pulmonary and systemic artery pressures was obtained, and this record was considered to represent the resting state. The patient's legs then were elevated and his feet attached to the pedals of a bicycle ergometer (10) which was positioned at the end of the fluoroscopy table. The revolutions of the ergometer caused the deflection of a galvanometer needle which was visible to the patient and was recorded photographically. In keeping the needle at a predetermined position a constant speed of rotation of the exercycle was maintained. After an average of four and one-half minutes from the start of exercise, blood samples again were withdrawn simultaneously from the pulmonary artery and radial artery, and from the inferior vena cava if a second catheter were in position. During a one minute interval, starting at the beginning of this sampling period (average rate of respirations: 25 per minute), the expired air was collected, the volume measured, and the gas analyzed by the Haldane method.

In an attempt to determine systemic blood flow during exercise, a blood sample was taken via catheter 2 from the inferior vena cava. Then this catheter was withdrawn

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to the superior vena cava and a second sample was taken; both samples were analyzed by the Van Slyke and Neill method and the results were averaged to give a measure of the oxygen content of mixed venous blood.

In the patients with patent ductus arteriosus in whom catheter 2 was not inserted, the tip of catheter 1 was withdrawn to the outflow, or midportion, of the right ventricle, and a further sample obtained. In patients with ventricular septal defect, the catheter was withdrawn from the pulmonary artery to the midportion of the right atrium and a sample was obtained from there.

Four patients were studied while breathing oxygen via a low-resistance, small dead-space system from a spirometer which contained approximately 35 liters of a mixture of 95 per cent oxygen and 5 per cent nitrogen at equilibrium with the subject's respiratory gases, and which incorporated a carbon dioxide absorber. The oxygen consumption was determined from the decrease in the volume of gas in the spirometer *1*) at rest for a period of three minutes, and *2*) during exercise for a period of one minute, centered on the time of collection of blood samples for manometric analysis.

Recordings of pulmonary and systemic artery pressures and sometimes of inferior and superior vena caval pressures during exercise were obtained immediately after the sampling of blood for Van Slyke analyses. The exercise then was discontinued.

Calculations. Among the data to be reported, the major emphasis will be placed on the values of pulmonary blood flow, pulmonary artery pressure, and total pulmonary resistance. Calculated values of systemic blood flow, systemic artery pressure and systemic vascular re-

TABLE I

Vital statistics and measured values from Group A: 16 patients with left-to-right shunts and severe pulmonary hypertension

Case no.*	Age (yr.) and sex	Surface area (M^2)	Diagnosis,† duration of exercise (min.)	Rest, exercise	Oxygen consumption (ml./min.)	Q_p‡ (L./min./M^2)	Q_s‡	P_{pa}‡ (mm. Hg)	P_{sa}‡	R_p‡ (dynes sec. cm.$^{-5}$)	R_s‡	Oxygen: Total content: Hemoglobin capacity (ml./100 ml. of blood)	Oxygen: Total content: Systemic artery	Oxygen: Total content: Pulmonary artery
1	43	1.60	VSD	R	279	3.6	3.7	121/53	119/65	1,050	1,120	24.7	22.4	19.7
	M		8	E	878	5.3	6.5	169/79	173/93	1,030	910	25.7	21.3	15.3
2	30	1.68	VSD	R	290	7.9	2.6	104/49	106/74	400	1,540	20.9	20.4	18.2
	M		7	E	768	7.3	4.9	133/56	147/78	530	960	20.7	19.4	14.1
3	34	1.85	VSD	R	216	1.9	2.7	130/73	135/83	2,180	1,590	27.0	23.7	19.9
	M		7	E	650	2.4	3.6	175/104	175/108	2,270	1,550	27.4	18.3	12.4
4	28	1.69	ASD	R	266	3.3	1.6	93/47	96/68	890	2,840	23.6	22.7	18.6
	M		5	E	667	4.0	2.3	115/61	122/84	890	2,510	24.3	21.5	14.4
5	14	1.23	VSD	R	205	6.9	4.2	126/76	137/70	880	1,420	18.2	18.1	15.7
	F		7	E	493	6.7	5.8	152/84	165/82	1,040	1,240	18.7	17.9	12.8
6	20	1.74	VSD	R	279	4.8	3.8	119/52	133/64	850	1,050	23.3	21.9	19.9
	M		8	E	865	4.9	6.8	167/78	165/84	1,010	790	24.0	19.1	13.7
7	15	1.22	VSD	R	179	7.3	3.7	121/68	139/65	760	1,600	18.2	18.0	16.0
	F		4	E	680	8.4		151/86	176/79	840		18.7	17.4	11.9
8	14	1.08	A-V canal	R	193	13.7	2.7	80/30	96/54	250	1,860	17.5	16.7	16.2
	M		5½	E	551	15.1		§	122/56	330		17.7	15.3	14.3
9	36	1.66	ASD	R	225	2.2	2.4	75/40	93/57	1,130	1,380	20.5	18.3	14.4
	M		7	E	424	1.9	2.1	118/55		1,930		21.9	‖	8.5
10	21	1.54	ASD	R	234	3.4	3.2	113/55	135/78	1,080	1,570	17.8	16.8	13.2
	F		5	E	563	3.7		148/64	163/83	1,320		18.0	13.0	7.6
11	37	1.73	ASD	R	273	3.7	2.8	74/3–7¶	110/68	450	1,320	21.6	20.6	16.9
	M		6	E	750	4.8	3.9	104/8–13¶	134/80		1,150	22.4	20.1	13.3
12	28	1.74	PDA	R	246	5.9	2.9	86/55	135/65	510	1,400	19.6	19.1	16.7
	F		9	E	615	6.8	6.4	110/69	169/87	550	820	19.6	19.3	14.1
13	34	1.93	ASD	R	208	7.2	3.4	76/34	120/68	280	1,040	21.9	21.5	20.0
	M		7	E	885	7.6		101/37	138/78	310		22.5	19.3	16.2
14	21	1.63	PDA	R	286	11.7	4.0	78/50	162/57	250	1,120	15.1	15.5	14.0
	F		5	E	667	9.6	5.3	104/63	170/66	390	960	15.4	15.9	11.6
15	44	2.38	PDA	R	290	3.0	2.1	76/35	160/81	550	1,740	21.1	20.6	16.5
	F		7	E	924	4.2	3.5	88/56	200/106	540	1,320	22.5	21.9	12.7
16	33	1.73	ASD	R	258	4.7	3.8	60/26	140/67	360	1,110	13.9	13.3	10.1
	M		6	E	721	5.8		93/41	165/70	460		14.6	14.1	6.9

* Cases arranged in order of decreasing pulmonary/systemic systolic pressure ratio at rest.

† VSD = Ventricular septal defect. ASD = Atrial septal defect. A-V canal = Atrioventricular canal. PDA = Patent ductus arteriosus.

‡ Q_p and Q_s = pulmonary and systemic blood flows. P_{pa} and P_{sa} = pulmonary and systemic arterial pressures, systole/diastole. R_p and R_s = pulmonary and systemic resistances.

§ Pulmonary artery mean pressure obtained.

‖ Oxygen saturation of 80 per cent by ear oximeter used in calculations.

¶ Right ventricular pressure. (Pulmonary artery pressure also obtained at rest.)

TABLE II

Vital statistics and measured values from Group B: 14 patients with left-to-right shunts but not severe pulmonary hypertension

Case no.*	Age (yr.) and sex	Surface area ($M.^2$)	Diagnosis,† duration of exercise (min.)	Rest, exercise	Oxygen consumption (ml./min.)	Q_p‡ (L./min./$M.^2$)	Q_s‡	P_{pa}‡ (mm. Hg)	P_{sa}‡	R_p‡ (dynes sec. cm.$^{-5}$)	R_s‡	Oxygen: Hemoglobin capacity (ml./100 ml. of blood)	Oxygen: Total content: Systemic artery	Oxygen: Total content: Pulmonary artery
17	43	1.50	ASD	R	212	16.0	4.7	32/10	134/64	60	970	17.7	17.7	16.8
	F		7	E	676	14.0		45/18	165/86	90		18.1	18.0	14.9
18	48	1.72	ASD	R	226	11.9	2.9	40/19	184/101	100	2,060	17.3	17.1	16.0
	F		8½	E	702	15.1		74/29	259/113	130		18.3	17.9	15.2
19	23	1.98	ASD	R	305§	9.0	2.8	27/14	110/72	80	1,150	20.7	21.4	19.7
	M		5	E	806§	10.4		30/12	124/77	70		21.0	22.1	18.2
20	25	1.59	ASD	R	244	8.5	3.3	25/10	117/68	90	1,260	16.8	17.0	15.2
	F		8	E	690	11.7	7.2	54/19	147/80	140	710	17.2	17.3	13.6
21	18	1.68	ASD	R	289	7.5	3.7	25/10	118/75	95	1,130	18.7	18.9	16.6
	M		6½	E	799	7.0		32/16	146/85	140		19.3	19.6	12.7
22	29	1.74	PDA	R	265§	7.2	4.0	21/10	118/63	110	870	16.6	18.2	16.1
	F		10	E	615§	6.2	4.8	15/10	135/68	100	650	16.6	17.9	12.2
23	20	1.63	ASD	R	231	6.8	3.4	26/11	118/69	120	1,320	19.1	19.2	17.1
	F		10	E	582	10.0	6.1	35/18	152/84	110	850	19.5	20.0	16.5
24	62	1.66	PDA	R	244	6.1	3.1	57/32	200/80	310	1,780	17.8	17.8	15.4
	F		8	E	569	6.6	5.5	72/40	216/91	370	1,150	18.3	18.5	13.3
25	47	1.47	PDA	R	256§	6.7	3.1	24/12	166/81	190	2,010	18.1	20.0	17.4
	F		10	E	525§	7.9	4.0	23/14	185/88	160	1,650	18.4	20.4	15.9
26	23	1.40	VSD	R	212	5.2	4.1	37/12	135/75	190	1,200	16.9	16.7	13.8
	F		7	E	769	8.2	6.8	44/15	133/65	170	740	17.3	17.4	10.7
27	18	1.44	VSD	R	247	5.2	3.4	26/11	132/64	220	1,630	20.1	20.1	16.8
	M		9	E	832	8.9	6.1	34/16	165/69	130	920	20.7	20.7	14.2
28	17	1.66	ASD	R	227	4.7	3.5	21/10	108/61	150	1,060	18.6	18.9	16.0
	M		9	E	1,149	10.0		47/25	158/85	150		19.5	19.2	12.3
29	31	1.96	PDA	R	304§	4.4	3.5	20/14	136/65	130	1,040	18.9	21.0	17.5
	M		9	E	1,123§	8.3	6.0	32/19	147/73	110	650	19.6	20.9	14.0
30	14	1.42	PDA	R	232	4.4	3.6	49/29	148/79	450	1,570	18.1	18.2	14.5
	M		6	E	667	6.3	5.8	53/25	158/81	340	1,040	18.3	18.4	11.0

* Cases arranged in order of declining pulmonary blood flow values at rest (first reading recorded in Q_p column).
† ASD = Atrial septal defect. PDA = Patent ductus arteriosus. VSD = Ventricular septal defect.
‡ Q_p and Q_s = pulmonary and systemic blood flows. P_{pa} and P_{sa} = pulmonary and systemic arterial pressures, systole/diastole. R_p and R_s = pulmonary and systemic resistances.
§ Estimate made while patient breathed 95 per cent oxygen.

sistance, and the magnitude of the intracardiac or great vessel shunts are reported also.

Pulmonary (Q_p) and systemic (Q_s) blood flows (liters per minute) were calculated by means of the Fick equation:

$$Q_p = \frac{\dot{V}_{O_2}}{C_{pv} - C_{pa}}, \quad Q_s = \frac{\dot{V}_{O_2}}{C_{sa} - C_{mv}},$$

where $\dot{V}_{O_2}$ is the oxygen consumption in cubic centimeters (S.T.P.D.) per minute, and the several C's in the denominators represent the oxygen contents in cubic centimeters per liter of blood from pulmonary vein, pulmonary artery and systemic artery, and mixed venous blood, respectively. In those subjects studied while breathing air, if the oxygen saturation of systemic artery blood was less than 95 per cent, the oxygen content of pulmonary vein blood was taken to be 98 per cent of the oxygen capacity plus 0.3 ml. per 100 ml. of blood to account for oxygen in physical solution while the subject breathed air. When the oxygen saturation of systemic artery blood exceeded 95 per cent, it was assumed that no right-to-left shunt existed, and the content of pulmonary vein blood (C_{pv}) was assumed to be equal to that of systemic artery blood (C_{sa}). The oxygen content of mixed venous blood (C_{mv}) was estimated in a different manner depending on the location of the left-to-right shunt as outlined previously. The assumptions pertaining to the use of such values will be discussed in detail in a later section.

The magnitudes of right-to-left (S_{r-l}) and left-to-right (S_{l-r}) shunts expressed as percentages, and the pulmonary (R_p) and systemic (R_s) resistances (pressure-flow ratios) expressed as dynes second cm.$^{-5}$ were calculated in the manner of Cournand, Baldwin, and Himmelstein (15).

$$S_{l-r} = \frac{C_{pa} - C_{mv}}{C_{pv} - C_{mv}} \times 100 \quad S_{r-l} = \frac{C_{pv} - C_{sa}}{C_{pv} - C_{mv}} \times 100.$$

A left-to-right shunt is thus expressed as the fraction of pulmonary artery blood originating from the pulmonary veins, and the right-to-left shunt is expressed as the

fraction of systemic artery blood originating from the cavae.

Patients. For this investigation 30 patients with single congenital intracardiac lesions characterized by left-to-right shunt were studied. In a few with pulmonary hypertension a small right-to-left shunt also was demonstrable while the patient was at rest. Of the 30 patients, 8 had a patent ductus arteriosus, 8 had a ventricular septal defect, 13 had an atrial septal defect, and 1 had a persistent common atrioventricular canal.

RESULTS

These patients were divided into two groups according to the presence, Group A (Table I), or absence, Group B (Table II), of severe pulmonary hypertension. It is difficult to select a specific value for pulmonary artery pressure above which pulmonary hypertension exists and below which it does not. In some patients the size of a ventricular septal defect or a patent ductus arteriosus is the effective limit to the volume of a veno-arterial shunt; in others the volume of pulmonary flow in relation to systemic flow is regulated chiefly by the response of the pulmonary vessels, and the size of the abnormal communication is not a major limiting factor. In this regard an apparently appropriate division between the patients in the present study was made by classifying those patients in whom the systolic pressure in the pulmonary artery at rest was 60 mm. Hg or more as having severe pulmonary hypertension. Those patients in whom the pulmonary artery systolic pressure was less than this value were considered not to have severe elevation of pulmonary artery pressure. The use of an arbitrary division such as this may result in the incorrect categorization of certain patients; but in the present group at least it appears that no patient without pulmonary hypertension of a severe degree has been included in Group A, while only two patients in Group B had pulmonary artery systolic pressures at rest in excess of 40 mm. Hg.

Data obtained by Barratt-Boyes and Wood (5) from a group of 12 normal subjects studied under similar conditions are included for purposes of comparison.

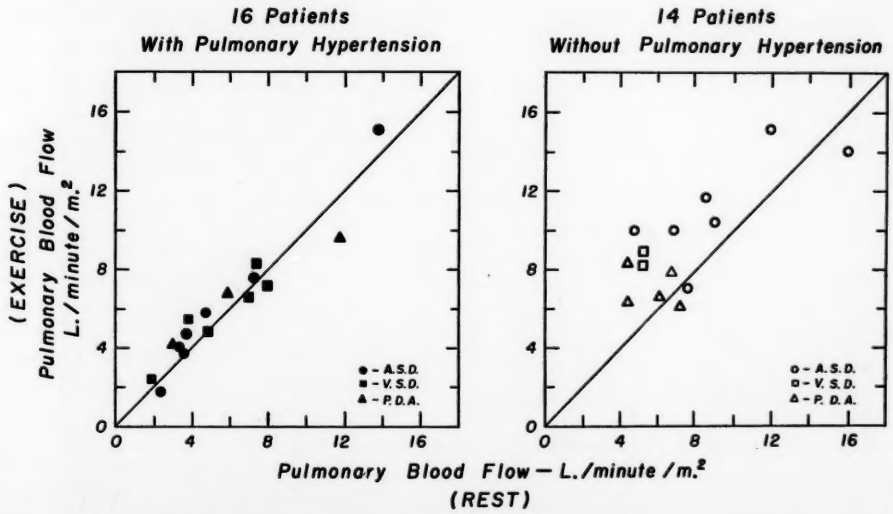

FIG. 1. THE RELATION OF PULMONARY BLOOD FLOW DURING EXERCISE AND AT REST

The 16 patients with severe pulmonary hypertension (Group A) are depicted by the solid symbols (left), and the 14 without severe pulmonary hypertension (Group B) by the open symbols (right). The diagonal indicates the line of identity. Note the high correlation ($r = 0.96$) and close absolute agreement of the values in Group A. These data can be expressed in the form of a regression equation, $Y = 0.97 + 0.91\ X$, in which X and Y are values at rest and during exercise, respectively. The standard error of the coefficient of regression is 0.07. Although there is a correlation between the values at rest and during exercise ($r = 0.74$) in Group B, it is less close than that in Group A.

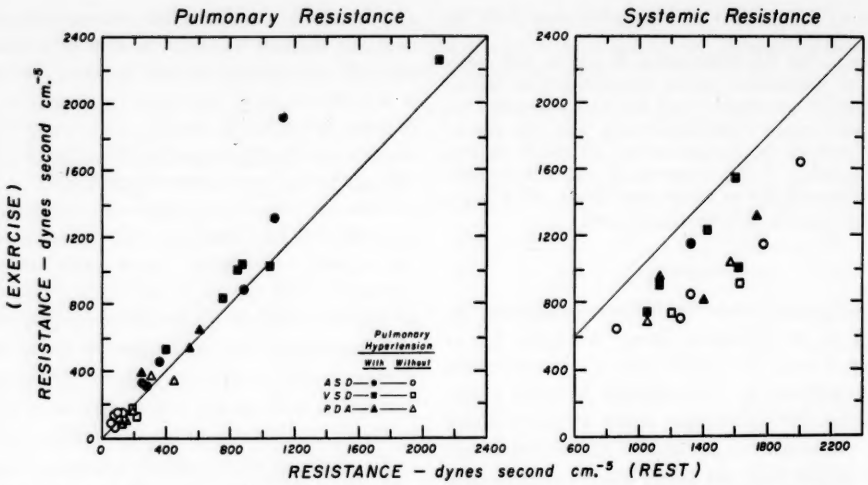

FIG. 2. THE RELATION OF PULMONARY AND SYSTEMIC PRESSURE FLOW RATIOS (VASCULAR RESISTANCES) OBTAINED DURING EXERCISE TO VALUES OBTAINED AT REST

The solid symbols indicate the patients with severe pulmonary hypertension. Pulmonary resistance values for 29 patients are depicted in the left panel. Correlation between values during exercise to those at rest is high ($r = 0.96$). The relationship can be expressed by the regression $Y = -5 + 1.23\,X$, where Y and X are values during exercise and at rest, respectively. The standard error of the regression coefficient is ± 0.09.

Systemic resistance values for 19 patients are shown in the right panel. The correlation between the values is 0.70, and the regression equation representing these data is $Y = 83 + 0.83\,X$.

Oxygen consumption and pulmonary blood flow, pressure, and resistance

Patients with severe pulmonary hypertension (Group A). The data for these patients are given in order of decreasing ratio of pulmonary to radial artery systolic pressures in Table I and in Figures 1, 2, and 3 (solid symbols). The average values and changes are summarized in Table III. The average increase in oxygen consumption for this group was significantly less than for the normal subjects (Table III), due at least in part to the inability of certain patients (Cases 5, 9, 10, and 14 in Table I) to maintain leg exercise at the desired level. There is no difference of hemodynamic significance related to the nature of the anatomic lesion present. The small increase in pulmonary blood flow of 0.47 liter per minute per M.^{2} which occurs during exercise does not appear to be a change of remarkable hemodynamic consequence. The correlation of 0.96 with a slope of regression of 0.91 between the pulmonary blood flow at rest and during exercise demonstrates the pulmonary flow to be unchanged for practical purposes in these patients during exercise.

Pulmonary artery pressures were increased in all cases measured. The highest absolute values of pulmonary artery pressures and pulmonary resistances both at rest (130/73 mm. Hg, 2,180 dynes second cm.$^{-5}$) and during exercise (175/104 mm. Hg, 2,270 dynes second cm.$^{-5}$) were obtained in Case 3; a slight decline in pulmonary resistance was seen in Case 15, the patient being a 44 year old woman with systemic hypertension and obesity in addition to patent ductus arteriosus.

Patients without severe pulmonary hypertension (Group B). The data for these patients are given in order of decreasing pulmonary blood flow in Table II and in Figures 1, 2, and 3 (open symbols). The average values and changes are summarized in Table III. The difference in oxygen consumption between the normal subjects and these patients was not significant.

The values for pulmonary blood flow during exercise are less well correlated ($r = 0.74$) with those obtained at rest than in Group A (Figure

1), and the increase in flow of 1.83 liters per minute per M.^{2} is highly significant ($p = 0.001$). Those patients in whom the resting pulmonary flow was less than 5.8 liters per minute per M.^{2} (that is, within the range of normal systemic flow for this laboratory) exhibited the greatest absolute and proportionate increase in blood flow. The changes in pulmonary artery pressure and pulmonary resistance are closely similar to the changes observed in the normal subjects.

Systemic blood flow and systemic resistance

In Group A, data for estimation of mixed venous blood were not obtained during exercise in three of the six cases of atrial septal defect, in one case of ventricular septal defect, nor in the patient with common atrioventricular canal. In Group B, no estimation of systemic blood flow was made during exercise in five of the seven cases of atrial septal defect. The average systemic blood flow (Table III) at rest was identical for the patients in Group B and for normal subjects, and slightly less for the patients in Group A. Three patients (Case Nos. 4, 9, and 15) had values outside the lower range of normal (2.5 liters per minute per M.^{2}). During exercise, the increase in systemic blood flow was somewhat less in Group A than in Group B or in the normal subjects. In both groups the changes in systemic artery pressure during exercise were similar to changes in the normal subjects.

The systemic resistance showed a diminution during exercise in both Group A and Group B, although it was of lesser magnitude in the former (Figure 2). In both groups the decline in systemic resistance was highly significant statistically.

Intracardiac or great vessel shunts

Intracardiac or great vessel shunts were calculated for all patients at rest and for the 11 patients

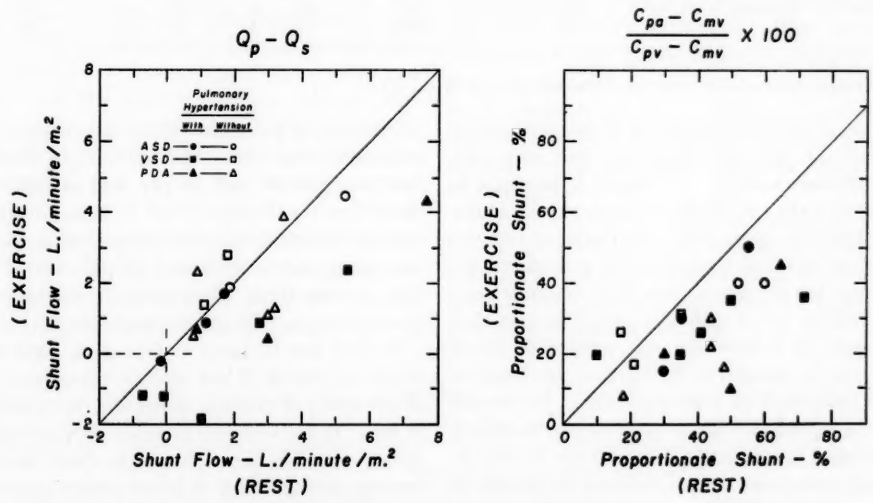

FIG. 3. COMPARISON OF THE MAGNITUDE OF INTRACARDIAC OR GREAT VESSEL SHUNTS AT REST AND DURING EXERCISE IN 20 PATIENTS

In the left panel the net shunt is expressed as the difference between pulmonary and systemic blood flows. Values to the left of and below the broken lines in the lower left corner indicate that systemic flow exceeds pulmonary flow and a net right-to-left shunt exists. Values to the right or above the broken lines indicate a net left-to-right shunt. Except in three patients (Cases 26, 27 and 29) with small communications, the volume of blood shunted in the left-to-right direction declined with exercise.

In the right panel the data are expressed as the proportion of pulmonary artery blood which is shunted in the left-to-right direction, and do not account for blood shunted right to left. All patients had a decline in the relative magnitude of the shunt on exercise except two. Of these, one patient (Case 29) had a small ventricular septal defect, and another (Case 3) had a large ventricular septal defect with severe pulmonary hypertension, and a shunt predominantly right to left both at rest and during exercise.

TABLE III

Average values and standard deviations

		Oxygen consumption (*ml./min./M.²*)	Heart rate (*beats/min.*)	Q_p (*L./min./M.²*)	Q_s	P_p S	P_p D	P_s S	P_s D (*mm. Hg*)	R_p	R_s (*dynes sec. cm.⁻⁵*)
Group A: 16 patients with pulmonary hypertension											
Rest	Average	148	84	5.70	3.10	96	51	126	68	760	1,417
	SD	23	17	3.30	0.64	23	14	21	8	500	229
Exercise	Average	416	121	6.17	4.69	129	67	159	82	896	1,169
	SD	280	21	3.16	1.47	29	18	22	13	578	367
Difference*	Average	+278	37	0.47	1.64	32	16	30	14	136	−255
	SD	65	23	0.93	1.20	10	8	12	9	226	300
Group B: 14 patients without pulmonary hypertension											
Rest	Average	151	81	7.34	3.50	31	15	137	73	164	1,360
	SD	22	16	3.25	0.50	11	7	28	10	106	383
Exercise	Average	455	115	9.17	5.81	42	20	164	82	158	968
	SD	112	16	2.95	0.93	17	8	36	12	88	327
Difference*	Average	+304	35	1.83	2.31	11	5	26	9	−6	−506
	SD	35	13	2.17	0.95	11	5	19	8	50	161
12 normal subjects †											
Rest	Average	141	72		3.50	21	11	148	77	189	1,290
	SD	14	10		0.80	3	2	13	7	59	265
Exercise	Average	479	111		5.70	31	15	177	86	162	889
	SD	58	15		1.10	6	5	19	10	36	59
Difference*	Average	+338	41		2.10	10	4	29	9	−23	−370
	SD	63	10		0.90	5	3	18	5	35	23

* Exercise values minus values at rest.
† From another similar study by Barrett-Boyes and Wood (5).

in Group A and the 9 in Group B during exercise in whom the systemic blood flow had been estimated during exercise. In Figure 3 the shunt is expressed as the net volume of blood passing in the left-to-right direction ($Q_p - Q_s$) and also as the proportion of blood traversing the pulmonary artery which has originated from the pulmonary vein. These values are of different significance in that the former is reduced by any volume of blood which may be shunted in the right-to-left direction and is expressed in absolute terms. In several cases the right-to-left shunt actually predominated and resulted in a negative value for the net shunt. A rough correlation exists between shunt values during rest and exercise as estimated by both methods.

For the patients with estimates of shunt both at rest and during exercise, the average net volumes of blood shunted left to right ($Q_p - Q_s$) were 2.3 liters per minute per M.² at rest and 0.8 during exercise in Group A, as compared to 2.6 at rest and 2.4 liters per minute per M.² during exercise in Group B. The differences between values at rest and during exercise were significant in Group A but not in Group B. Expressed as the proportions of pulmonary blood flow derived from pulmonary vein blood, the left-to-right shunts at rest averaged 44 and 46 per cent of pulmonary blood flow for Groups A and B, respectively, and showed a decline in each group during exercise to average values of 27 and 29 per cent (Figure 3). Among these 20 patients the changes were least in the five with atrial septal defects.

In 7 of the 16 cases in Group A, right-to-left shunts averaging 21 per cent (ranging from 10 to 40 per cent) of systemic blood flow were observed at rest. In the five with estimates both at rest and during exercise, the right-to-left shunt increased from an average value of 23 per cent to an average value of 34 per cent during exercise. In three additional patients in whom no right-to-left shunt was detected while the patient was at rest, right-to-left shunts averaging 12 per cent were demonstrable when the patient exercised. No right-to-left shunts were demonstrated for the patients in Group B.

COMMENT

Donald, Bishop, Cumming, and Wade (4) found in normal subjects that the major changes

in both the oxygen consumption and the arteriovenous oxygen difference were virtually complete after two minutes of exercise, and they observed no further change in these variables when exercise was continued at the same level. Even in the majority of patients with rheumatic heart disease, the alterations in these variables are essentially complete after five minutes of exercise at a uniform rate, and the subject is in a steady state (6). The majority of our patients exercised for 7 to 12 minutes, and observations were made in the fourth to sixth minutes. For some of the more disabled patients the period of exercise was shorter, and observations were commenced at approximately two and one-half minutes and completed by the fourth or fifth minute. If a steady state had not been attained when observations were begun, significant changes would occur in the oxygen saturation of blood samples drawn during the period of observation of them. Measurement, therefore, was made of the continuous photographic record of the oxygen saturation of the blood during its withdrawal from the radial and pulmonary arteries through cuvette oximeters into syringes for manometric analysis, and the difference in oxygen saturation during the initial and the final 10 second periods of sampling was determined (Table IV). The interval between these periods was 20 to 35 seconds. The changes observed were usually very small and there was no consistent difference between the beginning and end of the sampling period. The greater variation observed occurred among patients in Group B who had exercise for periods in excess of six minutes.

Pulmonary blood flow measurement

One of the main purposes of this study is to document the effect of exercise on pulmonary blood flow. If the estimate of oxygen consumption during the exercise period is assumed valid—and there is no reason to doubt this assumption—then the measurement of pulmonary flow depends on accurate values for the oxygen content of pulmonary vein and pulmonary artery blood.

The samples from the pulmonary artery and from a systemic artery always were drawn simultaneously. In all cases the assumption was made that there was no impairment of oxygenation of pulmonary capillary blood. No patients in Group B showed desaturation of systemic arterial blood during exercise. Furthermore, in a great majority of patients with cyanosis and intracardiac defects studied in this laboratory—including some patients in Group A while at rest—the quantitative estimates of right-to-left shunts by indicator techniques have agreed well with the estimates based on the oxygen saturation values, thus militating against desaturation of pulmonary vein blood as the basis for desaturation of systemic artery blood in such patients.

As for the oxygen content of pulmonary artery blood, it is usual to regard samples of blood withdrawn from the first chamber distal to the site of shunt as representing adequately the oxygen saturation of the blood entering the lungs. In the

TABLE IV

Differences between initial and later oxygen saturation values at rest and during exercise

	Change in per cent of oxygen saturation*			
	Rest		Exercise	
	Radial artery	Pulmonary artery	Radial artery	Pulmonary artery
Group A				
Average change†	0	−0.22	−0.15	−0.33
Range	(+1.5 to −0.6)	(+0.5 to −1.1)	(+0.7 to −1.0)	(+0.5 to −1.0)
Group B				
Average change†	−0.25	−0.19	+0.13	+0.22
Range	(+0.2 to −1.3)	(+0.7 to −1.8)	(+1.0 to −0.8)	(+4.6 to −3.5)

* Saturation values measured by cuvette oximeter and recorded over periods of 40 to 55 seconds as samples were drawn at rest and in the fourth to sixth minutes of exercise.

† Mean value for final 10 seconds of blood flow minus mean value for initial 10 seconds of blood flow.

cases of patent ductus arteriosus studied in this laboratory, the oxygen saturation of blood samples obtained from different locations in the right and left pulmonary arteries has been strikingly uniform, which implies that mixing of blood occurs to a sufficient degree in the pulmonary artery so that a sample of pulmonary artery blood is representative of all the blood entering the lungs.

Calculation of systemic blood flow and intracardiac and great vessel shunts

A major difficulty was the determination of an accurate value for mixed venous blood. In this study potential errors in the estimation of oxygen content of mixed venous blood during exercise would appear to be smallest in the eight cases of patent ductus arteriosus in which the blood samples were obtained from the outflow, or midportion, of the right ventricle, and greatest in the five cases of atrial septal defect in which samples of blood were obtained from the inferior and superior venae cavae (Table V). Data obtained in Case 18 also are included in this table, but calculations of systemic blood flow and intracardiac shunts were not made because examination of a roentgenogram taken at the time of sampling intended to be from the inferior vena cava clearly showed the catheter tip to be in an hepatic vein. Calculation of the systemic blood flow was not attempted in the remaining seven cases of atrial septal defect. In Cases 4, 9, and 11 the wide differences between arterial and venous blood and the relatively small differences between inferior and superior caval blood serve to reduce potential errors. In Case 20 the possibility of preferential sampling of hepatic vein blood is suggested by the higher saturation of inferior caval blood compared to superior caval blood, whereas in Case 23 the relatively small arteriovenous difference associated with a considerable superior-inferior caval difference makes possible a large error. To test this further, for these five cases the magnitude of the left-to-right shunt during exercise was recalculated using the lowest saturation value obtained to represent mixed venous blood. On the average, a decline in the shunt was still present even in this more extreme circumstance.

TABLE V

Six patients with atrial septal defect

Case No.	Oxygen saturation (*per cent*)			
	Rest		Exercise	
	IVC*	SVC†	IVC*	SVC†
4	60	53	18	21
9	65	59	37	42
11	74	66	37	47
18	80	74	74‡	47
20	78	68	71	60
23	83	76	60	85

* IVC = Inferior vena cava.
† SVC = Superior vena cava.
‡ Catheter tip in hepatic vein during exercise.

As a further test of the validity of the calculations of systemic blood flow, the relation of the increase in blood flow to the increase in oxygen consumption during exercise was examined. In Case 20 the calculated increase in blood flow was 1,300 ml. per minute for each 100 ml. per minute of increase in oxygen consumption, and in Case 23 the corresponding increase was 1,200 ml. per minute. These values greatly exceed the average of 626 ± 217 ml. per minute obtained in the normal subjects. In the complete series of patients, four cases (Nos. 12, 20, 23, and 24) produced calculated systemic blood flow values which were disproportionate to the increase in oxygen consumption. Exclusion of these four cases would alter the reported increase in systemic blood flow during exercise by only 0.10 liter per minute per $M.^2$ in Group A, and 0.21 liter per minute $M.^2$ in Group B, and would not alter the significance of the decline in left-to-right shunt which occurred during exercise. While the considerations just presented weaken the quantitative evaluation of figures for systemic blood flow and shunt obtained during exercise, the directional changes already suggested appear valid.

Changes in pulmonary vascular dynamics

Patients with pulmonary hypertension. The close relation between pulmonary blood flow values obtained at rest and during exercise deserves further comment. In the presence of pulmonary hypertension, the pulmonary blood flow appears to be relatively fixed (Figure 1), although a small increase was demonstrated during exercise. Bruce and John (8) reported that arterial desaturation occurred in two of their patients, but they did not report a fixed pulmonary blood flow. It

is not possible to state that the increase in calculated resistance represents a change in the pulmonary blood vessels; however, the small change in left atrial flow with exercise makes changes in left atrial pressure unlikely. The analogy of changes in pulmonary blood vessels to the behavior of a linear potentiometer in a simple D.C. circuit may be quite misleading in instances such as these; here the impedance of a frequency dependent complex circuit may in fact be the more appropriate model.

As would be expected, apart from Case 4, in which evidence of cardiac failure was present, the systemic vascular resistance declined during exercise in every patient in whom the comparative measurements were made, and to about the same degree as in normal subjects. Difficulties of estimation of systemic blood flow notwithstanding, these data clearly indicate the difference in the responses of the pulmonary and systemic circulations produced by muscular activity.

The direction and magnitude of a shunt through a large ventricular septal defect or a patent ductus arteriosus are determined principally by the relative resistances offered by the pulmonary and systemic circulations to the flow of blood from the ventricles. A useful expression of these relative resistances is a ratio of pulmonary to systemic resistance, which minimizes the effect of nonspecific factors such as body size and allows comparison from subject to subject. In this group of patients, the average resistance ratio of 0.50 at rest was increased to 0.74 during exercise, owing mainly to the fall in systemic resistance. In cases with severe pulmonary hypertension and a level of pulmonary blood flow not above the range of normal while at rest (example: Case 6), the change in the pulmonary/systemic resistance ratio during exercise may result in a shunt predominantly in the right-to-left direction. The data suggest that cases of atrial septal defect with pulmonary hypertension may respond in a somewhat similar fashion; presumably there may be impedance of right ventricular filling due to a raised right ventricular diastolic pressure consequent on the increased pulmonary resistance. Alternatively, facilitation of left ventricular filling may be a result of the lowered systemic vascular resistance.

Patients without severe pulmonary hypertension. In the patients of Group B there was a definite increase in pulmonary blood flow during exercise as compared to that during rest, but in no patient did it rise to the level reported by Bruce and John (8) for upright walking. As in Group A, a lesser proportion of pulmonary blood flow is composed of pulmonary vein blood during exercise in cases with a large increase in resting pulmonary flow. In those cases with a small patent ductus arteriosus or ventricular septal defect, the size of the abnormal communication and the left ventricular or aortic pressure should logically be the principal determinants of the small shunt that occurs. The absolute and even the relative magnitude of the shunt has been demonstrated to increase during exercise in such cases.

General interpretations

Excluding those patients without pulmonary hypertension and without a shunt of functional significance in the left-to-right direction, the general response of the pulmonary vascular bed to exercise in the supine position appears to permit but a small increase in pulmonary blood flow, with an increase in pulmonary resistance in those patients who have pulmonary hypertension. There is usually a concomitant decrease in the absolute and relative magnitude of the left-to-right shunt, apparently related to the large decrease in the systemic vascular resistance which occurs uniformly during exercise. The consequences of these responses are of some significance, since it is clear that patients with large left-to-right shunts may adapt to the circulatory needs of exercise without major change in pulmonary vascular dynamics. During activity, the useful work of the right ventricle appears to be increased in that a greater absolute and relative amount of the output of this chamber is composed of systemic venous blood. This may explain the relatively poor correlation of physical development and exercise tolerance of many patients with congenital heart disease on the one hand, with the degree of alteration in circulatory dynamics when such patients are studied in the supine position on the other. In certain of the more disabled patients with severe pulmonary hypertension, this response may circumvent a stress of potentially fatal magnitude on the circulation such as does occur in the postoperative period following closure of patent

ductus arteriosus without relief of severe pulmonary hypertension (16).

These responses produce in general a favorable situation for patients with left-to-right shunts, since the demands on the heart for an increase in systemic blood flow to meet the requirements of exercise are met without a concomitant increase in stress on those portions of the central circulation already overburdened by the presence of the abnormal shunt.

SUMMARY

1. The changes in pulmonary vascular dynamics associated with exercise in the supine position were studied in 30 patients with intracardiac or aortopulmonary left-to-right shunt. The patients were subdivided into Group A, whose pulmonary artery systolic pressures exceeded 60 mm. Hg at rest, and Group B, whose pulmonary artery systolic pressures at rest were less than 60 mm. Hg. Group A was composed of seven patients with atrial septal defect (including one with common atrioventricular canal), six with ventricular septal defect, and three with patent ductus arteriosus; Group B was composed of seven patients with atrial septal defect, two with ventricular septal defect, and five with patent ductus arteriosus.

2. The pulmonary blood flow averaged 5.70 liters per minute per M.^{2} for Group A at rest, and increased to 6.17 liters per minute per M.^{2} during exercise. There was a strong correlation ($r = 0.96$) between the flow values at rest and during exercise. For Group B the average pulmonary blood flow at rest of 7.34 liters per minute per M.^{2} was increased to 9.17 liters per minute per M.^{2} during exercise; but the correlation was less close ($r = 0.74$) than for Group A.

3. Pulmonary artery pressure was increased during exercise in both groups. The pulmonary resistance was significantly elevated in Group A from an average value of 760 dynes second cm.$^{-5}$ at rest to 896 dynes second cm.$^{-5}$ during exercise. In contrast, there was no consistent increase in pulmonary resistance during exercise in Group B. Absence of data concerning pulmonary artery wedge (left atrial) pressure precludes a precise statement as to changes in pulmonary arteriolar resistance.

4. Systemic blood flows were calculated in 20 of the patients during exercise. The increase in systemic blood flow during muscular activity was greater than the increase in pulmonary blood flow. In contrast to the increase in pulmonary resistance in Group A, the normal decline in systemic vascular resistance associated with exercise was observed in both groups.

5. The average magnitude of left-to-right shunts declined in Group A from 44 per cent of the pulmonary blood flow at rest to 27 per cent during exercise, and in Group B from 46 per cent to 29 per cent. In seven patients, right-to-left shunts were present at rest, and these shunts increased in magnitude during exercise in the five patients from whom quantitative data were obtained during the latter state. In three other patients, right-to-left shunts developed during exercise, although no such shunt was detected at rest.

6. The differing responses of the pulmonary and the systemic circuits to exercise are considered to be the underlying basis for the relatively small change in pulmonary flow during exercise in patients with large defects. In consequence, the increased demands placed on the heart during exercise are met with relatively little additional stress on those parts of the circulation already overburdened by the presence of the left-to-right shunt.

REFERENCES

1. Riley, R. L., Himmelstein, A., Motley, H. L., Weiner, H. M., and Cournand, A. Studies of the pulmonary circulation at rest and during exercise in normal individuals and in patients with chronic pulmonary disease. Amer. J. Physiol. 1948, **152**, 372.
2. Hickam, J. B., and Cargill, W. H. Effect of exercise on cardiac output and pulmonary arterial pressure in normal persons and in patients with cardiovascular disease and pulmonary emphysema. J. clin. Invest. 1948, **27**, 10.
3. Dexter, L., Whittenberger, J. L., Haynes, F. W., Goodale, W. T., Gorlin, R., and Sawyer, C. G. Effect of exercise on circulatory dynamics of normal individuals. J. appl. Physiol. 1951, **3**, 439.
4. Donald, K. W., Bishop, J. M., Cumming, G., and Wade, O. L. The effect of exercise on the cardiac output and circulatory dynamics of normal subjects. Clin. Sci. 1955, **14**, 37.
5. Barratt-Boyes, B. G., and Wood, E. H. Hemodynamic response of healthy subjects to exercise in the supine position while breathing oxygen. J. appl. Physiol. 1957, **11**, 129.
6. Ferrer, M. I., Harvey, R. M., Cathcart, R. T., Cournand, A., and Richards, D. W., Jr. Hemodynamic studies in rheumatic heart disease. Circulation 1952, **6**, 688.

7. Donald, K. W., Bishop, J. M., and Wade, O. L. A study of minute to minute changes of arteriovenous oxygen content difference, oxygen uptake and cardiac output and rate of achievement of a steady state during exercise in rheumatic heart disease. J. clin. Invest. 1954, **33**, 1146.
8. Bruce, R. A., and John, G. G. Effects of upright posture and exercise (grade walking) on pulmonary hemodynamics in patients with left-to-right shunts (abstract). Circulation 1956, **14**, 916.
9. Swan, H. J. C., Marshall, H. W., and Wood, E. H. The effect of exercise on the pulmonary blood flow in patients with left-to-right shunts (abstract). J. Lab. clin. Med. 1956, **48**, 949.
10. Wood, E. H. Special technics of value in the cardiac catheterization laboratory. Proc. Mayo Clin. 1953, **28**, 58.
11. Wood, E. H. Oximetry *in* Medical Physics, Otto Glasser, Ed. Chicago, The Year Book Publishers, Inc., 1950, vol. 2, pp. 664–680.
12. Van Slyke, D. D., and Neill, J. M. The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. I. J. biol. Chem. 1924, **61**, 523.
13. Sendroy, J., Jr. Manometric determination of hemoglobin by the oxygen capacity method. J. biol. Chem. 1931, **91**, 307.
14. Roughton, F. J. W., Darling, R. C., and Root, W. S. Factors affecting determination of oxygen capacity, content and pressure in human arterial blood. Amer. J. Physiol. 1944, **142**, 708.
15. Cournand, A., Baldwin, J. S., and Himmelstein, A. Cardiac Catheterization in Congenital Heart Disease: A Clinical and Physiological Study in Infants and Children. New York, The Commonwealth Fund, 1949.
16. Ellis, F. H., Jr., Kirklin, J. W., Callahan, J. A., and Wood, E. H. Patent ductus arteriosus with pulmonary hypertension: An analysis of cases treated surgically. J. thorac. Surg. 1956, **31**, 268.

GLUTATHIONE REDUCTASE ACTIVITY IN BLOOD AND BODY FLUIDS[1]

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Reduced glutathione (GSH) is readily oxidized by the cytochrome systems of animal tissues (1). This oxidation is reversed by the action of an enzyme, glutathione reductase (GR), which catalyzes the reduction of oxidized glutathione (GSSG) by reduced triphosphopyridine nucleotide (TPNH).

$$TPNH + GSSG + H^+ \rightleftharpoons TPN + 2GSH$$

It has been suggested that GR is largely responsible for the ability of living tissues to keep glutathione primarily in the reduced form, a condition which may be important in the maintenance of sulfhydryl enzymes in the active state (2).

It is the purpose of this report to present the findings of the study designed to determine the presence and significance of GR in human and animal blood components and in other body fluids.

METHODS AND MATERIALS

Glutathione reductase activity may be measured by various techniques (3), including a spectrophotometric assay. Spectrophotometrically, GR activity is determined by following at 340 mμ the decrease in optical density of a reaction mixture containing TPNH. The optical density change reflects the conversion of TPNH to TPN during the course of the reduction of oxidized glutathione by GR.

Oxidized glutathione, TPNH, and bovine serum albumin were obtained commercially and were used without further purification.

Oxidized glutathione was prepared for use by dissolving 200 mg. in 5.0 ml. of distilled water; the solution was prepared daily. TPNH was prepared by dissolving 20 mg. in 3.0 ml. of buffer, and further dilution with buffer was made in order to have a final solution of optical density between 750 and 850 at 340 mμ; this solution was also prepared daily. Bovine serum albumin, free of GR activity, was used in amounts of 50 mg. dissolved in 10.0 ml. of buffer. The buffers (0.15 M at pH 7.6) were either potassium dibasic phosphate or tris (hydroxymethyl) aminomethane (Tris).

Serum, plasma with heparin, citrate or oxalate, and heparinized whole blood were obtained by venipuncture, without regard to the fasting state. Oxalated plasma showed consistently lower activities and was not used for the study. Serum and plasma were separated by centrifuging at 2,000 r.p.m. for ten minutes at room temperature. Unless otherwise stated, all blood and body fluids samples were refrigerated at 4° C. within three hours after collection and were used for GR assay within three days after collection. Control studies demonstrated that enzyme activity was not appreciably changed during these lengths of time at room temperature. Cerebrospinal fluid specimens were obtained by spinal tap between lumbar vertebrae 4 and 5. Specimens were centrifuged at 2,000 r.p.m. for 10 minutes at room temperature and the supernatant was used for GR determination. Serous effusions were obtained by the usual technique of thoracentesis or paracentesis and the specimens handled as described for cerebrospinal fluids. Lysed red cells were prepared by using 0.1 ml. of heparinized packed erythrocytes which was diluted with 5.0 ml. of distilled water and centrifuged at 2,000 r.p.m. for 10 minutes to remove ghosts.

Tissue specimens were obtained during the course of postmortem examination and within five hours of death. The tissues were used within two hours after sampling, and were prepared for GR assay by homogenizing with normal saline, at room temperature. The loss of activity after death and during the two hours after sampling was not estimated.

The patients from whom body fluid specimens were obtained were either supervised clinically and/or were evaluated by a review of the clinical records by the authors. The stated diagnoses were arrived at on the basis of the clinical and/or postmortem findings.

Glutathione reductase activity was determined spectrophotometrically. To 0.5 ml. serum or plasma were added 2.3 ml. of phosphate buffer and 0.1 ml. of TPNH. The mixture was incubated at room temperature (26° C.) for 20 minutes after which 0.1 ml. of GSSG was added. The reaction was followed in a model DU Beckman spectrophotometer using a corex cell with 1 cm. lightpath. At 15 or 30 second intervals, five consecutive readings were made of the change in optical density. From the average decrease in optical density during five 15 or 30 second intervals, the change in optical density per minute per ml. of serum or plasma was calculated.

[1] The initial phases of this work were done at Irvington House, Irvington-on-Hudson, N. Y., under a grant from the New York Heart Association.

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TABLE I

The effect of heat on glutathione reductase *

Duration of time heated (*min.*)	Serum GR activity (*U/ml./min.*)
0	160
5	80
10	60
15	40
60	12

* Serum was obtained from a patient with acute toxic hepatitis and heated in a water bath for different lengths of time at 56° C.

One unit of GR activity is defined as a change in optical density of 0.001 per ml. per minute at 340 mμ.

The same procedure was followed for the measurement of GR activity in the case of serous effusion, lysed red cells, and cerebrospinal fluid, except that 0.2 ml. of bovine albumin was added to the incubation mixture and the amount of phosphate buffer was reduced to 2.1 ml. (4).

RESULTS

Some properties of serum glutathione reductase

Sera kept frozen for one week at −10° C., or kept in the refrigerator at 4° C. for an equal length of time, showed no significant decrease in activity.

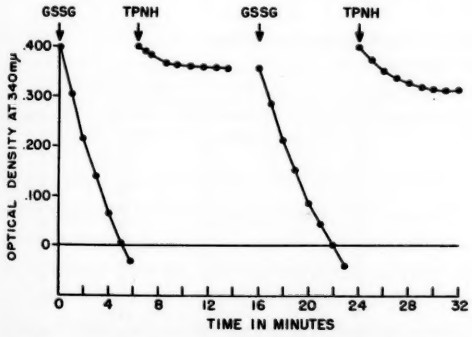

FIG. 1. THE CHANGE IN OPTICAL DENSITY WHICH RESULTS WHEN SERUM GLUTATHIONE REDUCTASE IS MEASURED AS DESCRIBED IN THE TEXT

At zero time, the reaction was begun by adding 0.1 ml. of GSSG to the reaction mixture of phosphate buffer, serum, and TPNH. The change in optical density was linear for two minutes and then slowed in rate. At six minutes, the decrease in optical density was no longer perceptible. At that time, 0.1 ml. TPNH was added and reaction resumed but proceeded slowly and terminated before the fourteenth minute. When the optical density no longer changed, 0.1 ml. GSSG was added and the reaction resumed and the change in optical density was linear until the twenty-third minute.

TABLE II

Glutathione reductase activity in human tissue homogenates *

Tissue homogenates	GR activity (*U/ml./min./Gm. of wet tissue*)
Liver	3,200
Kidney	3,200
Pancreas	1,300
Heart	600
Thyroid	260
Lysed erythrocytes	200
Spinal cord	160
Lung	120
Serum	40

* In each instance the values given are the average of two tissue determinations except for spinal cord and thyroid tissues, in which cases only one specimen of each was examined.

Heating sera at 56° C. for five minutes resulted in a decrease in GR activity of approximately 50 per cent, whereas heating for a longer period of time caused further decrease in activity (Table I).[3] Other investigators found a definite decrease in GR activity of plant tissue, following dialysis for a period of 17 to 72 hours and against a phosphate buffer of 0.025 M at pH 6.2 (5). In the present study no significant decrease in GR activity was noted when sera were dialyzed against 0.15 M phosphate buffer pH 7.6 at 4° C. for eight hours. Addition of manganous salts in a concentration of 0.4 times 10^{-4} M per test did not affect the activity of the serum (5).

Initial studies were done with Tris buffer; how-

TABLE III

Glutathione reductase activity of heparinized plasma and lysed erythrocytes of 10 species of animals

Animal	GR activity	
	Plasma (*U/ml./min.*)*	Lysed erythrocytes (*U/ml. packed cells/min.*)*
Hamster	70	200
Rabbit	40	600
Guinea pig	27	100
Monkey	26	300
Goat	16	60
Mouse	16	50
Chicken	8	200
Dog	6	200
Pig	4	800
Rat	4	80

* One sample of each was studied.

[3] Two experiments were done on two different sera with approximately the same results.

ever, GR activity was found to be approximately twice as high on phosphate buffer as compared to Tris buffer. Optimal phosphate buffer osmolarity for the enzyme reaction was found to be 0.15 M. No significant difference in GR activity was noted between pH 8.1 to 7.3, although a decided decrease in GR activity was observed when the pH of the reaction mixture went beyond these values.

If the enzymatic reaction was followed spectrophotometrically until no further change in optical density occurred, the reaction resumed upon the addition of oxidized glutathione attesting to the presence of excess TPNH and to the attainment of a chemical equilibrium (Figure 1).

Glutathione reductase activity of blood

Serum and heparinized plasma have similar GR activity. Slight degrees of hemolysis did not significantly influence the enzymatic activity of serum or plasma. Sera obtained from 98 normal individuals ranging in age from 10 to 60 years had a mean activity of 40 units per ml. per minute, with a standard deviation of 15 units. Using two standard deviations, the normal range of activity for human serum is 10 to 70 units. No significant difference in serum GR activity was noted in regard to the fasting state, sex, age or color. Table II compares the GR activity of human tissue homogenates, including lysed erythrocytes.

The GR activity of heparinized plasma and lysed erythrocytes from 10 species of animals is presented in Table III.

Glutathione reductase activity of body fluids obtained from patients with various disease states

Although a wide spectrum of patients with various disease states was examined, abnormally increased serum GR was noted in patients with acute hepatic disease and in patients with carcinoma. Table IV lists the serum GR activity in 104 diseased individuals studied. Figure 2 shows the serial alterations of serum GR activity in a patient with acute homologous serum hepatitis. Although only a limited number of cerebrospinal fluids and serous effusions were examined (Tables V and VI), apparently increased GR activity was

TABLE IV

Serum glutathione reductase activity in patients with various disease states

Diagnosis	No. of patients	No. of samples	GR activity		
			10–70	71–200	201–700
Viral hepatitis (1st week)	7	26	0	25	1
Hepatic coma	1	2	0	1	1
Laennec's cirrhosis	4	4	4	0	0
Lung tuberculosis	2	2	2	0	0
Systemic lupus erythematosus	2	3	1	0	2
Acute ileitis	1	1	1	0	0
Acute gout	1	5	5	0	0
Toxic hepatitis due to salicylates	4	8	5	3	0
Acute rheumatic fever	3	9	9	0	0
Acute myocardial infarct (1st week)	5	15	15	0	0
Coronary insufficiency	1	2	2	0	0
Congestive heart failure	3	3	3	0	0
Multiple sclerosis	1	1	1	0	0
Hypoparathyroidism	1	1	1	0	0
Macroglobulinemia	1	2	2	0	0
Acute glomerulonephritis	1	2	2	0	0
Pyelonephritis	1	1	1	0	0
Bilateral kidney infarcts	1	1	0	1	0
Infectious mononeucleosis	1	3	3	0	0
Diabetes	2	3	3	0	0
Gaucher disease	1	1	0	1	0
Iron deficiency anemia	2	2	2	0	0
Carcinomas	38	55	11	31	13
Sarcomas	6	7	5	2	0
Hodgkin's disease	7	13	13	0	0
Leukemia	6	7	7	0	0
Thymoma	1	1	1	0	0
Total	104	180			

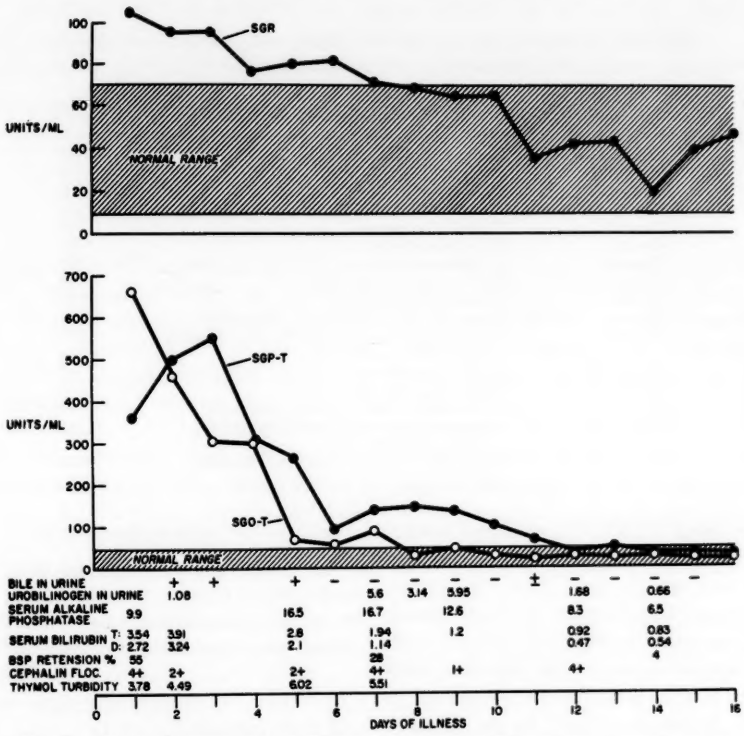

FIG. 2. SERIAL ALTERATIONS OF SERUM GLUTATHIONE REDUCTASE ACTIVITY IN A PATIENT WITH ACUTE HOMOLOGOUS SERUM HEPATITIS

Serum glutathione reductase activity, serum glutamic oxalacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT) were followed for 16 days, together with liver function tests.

noted only in cerebrospinal fluid from individuals with acute bacterial meningitis and in serous effusions containing malignant cells. It is pertinent to note that patients with elevated GR activity in pleural or peritoneal fluids did not necessarily have elevated serum activity of the enzyme.

SUMMARY AND CONCLUSIONS

Body fluid and tissue homogenate GR activity can be readily measured spectrophotometrically. All human sera, erythrocytes, tissue homogenates, heparinized plasma, and ten different animal samples of plasma and erythrocytes examined had GR

TABLE V

Cerebrospinal fluid glutathione reductase activity in patients with various central nervous system disease states

Diagnosis	No. of patients	GR activity (*U/ml./min.*)			
		0–5	6–10	11–20	21–33
Ruptured lumbar disc	5	4	1	0	0
Cerebral concussion	1	1	0	0	0
Migraine	1	1	0	0	0
Possible colitis	1	1	0	0	0
Acute labyrinthitis, diffuse	1	0	1	0	0
Hydrocephalus	1	1	0	0	0
Cerebral hemorrhage	2	1	1	0	0
Meningococcic meningitis	1	0	0	1	0
Tuberculous meningitis	1	0	0	0	1

TABLE VI

Glutathione reductase activity of pericardial, pleural, and peritoneal serous effusions

Patient No.	Cavity site of effusion	Diagnosis	Effusion GR activity	Serum GR activity
Ef 1	Pericardial	Acute idiopathic pericarditis	32	60
Ef 2	Pleural	Pneumonitis	8	20
Ef 3	Pleural	Pneumonitis	16	34
Ef 4	Pleural	Hodgkin's disease	22	
Ef 5	Pleural	Hodgkin's disease	24	
Ef 6	Pleural	Lymphosarcoma	24	80
Ef 7	Pleural	Lymphosarcoma	60	
Ef 8	Pleural	Reticulum cell sarcoma	60	90
Ef 9	Pleural	Malignant lymphoma	12	24
Ef 10	Pleural	Adenocarcinoma of breast, metastatic	2,100	32
Ef 11	Pleural	Adenocarcinoma of unknown origin, malignant cells in effusion	300	32
Ef 12	Peritoneal	Laennec's cirrhosis	5	
Ef 13	Peritoneal	Laennec's cirrhosis	6	60
Ef 14	Peritoneal	Adenocarcinoma of colon, metastatic	260	132
Ef 15	Peritoneal	Adenocarcinoma of breast, metastatic and lymphosarcoma	1,760	16
Ef 16	Peritoneal	Adenocarcinoma of uterus, metastatic	56	80

activity. The mean GR activity of normal human sera is 40 ± 15 units per ml. Normal human erythrocytes have two to three times the GR activity of the respective plasma. Increased serum GR activity was noted primarily in patients with homologous serum and infectious hepatitis, toxic hepatitis due to salicylates, hepatic coma, and in patients with carcinoma usually in the disseminated phase of the disease; patients with lymphomata do not appear to have increased serum GR activity. The only individuals with appreciable cerebrospinal fluid GR activity are those with acute bacterial meningitis. Serous effusions with appreciable GR activity were noted to have malignant cellular constituents. Extension of these observations on the relationship of GR activity of serum, cerebrospinal fluid and serous effusions to various disease states will be necessary in order to evaluate the clinical significance of alterations of GR activity in body fluids.

ACKNOWLEDGMENT

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REFERENCES

1. Fruton, J. S., and Simmonds, S. General Biochemistry. New York, John Wiley and Sons, Inc., 1953, p. 343.
2. Conn, E. E., and Vennesland, B. Glutathione reductase of wheat germ. J. biol. Chem. 1951, 192, 17.
3. Colowick, S. P., and Kaplan, N. O. Methods in Enzymology. New York, Academic Press Inc., 1955, vol. II, p. 719.
4. Racker, E. Glutathione reductase from bakers' yeast and beef liver. J. biol. Chem. 1955, 217, 855.
5. Mapson, L. W., and Goddard, D. R. The reduction of glutathione by plant tissues. Biochem. J. 1952, 49, 592.

STUDIES WITH BRUCELLA ENDOTOXIN IN HUMANS: THE SIGNIFICANCE OF SUSCEPTIBILITY TO ENDOTOXIN IN THE PATHOGENESIS OF BRUCELLOSIS

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The mechanisms by which patients ill with brucellosis develop their characteristic symptoms of fever, chills, myalgia, asthenia and malaise have not been precisely elucidated. In particular, the contributions of the brucella cell itself to the pathogenesis of these clinical features have not been extensively investigated. An unusual clinical observation (1) suggested that some component of the cell did play a significant role. When patients seriously ill with brucellosis due to *Brucella melitensis* were treated with chlortetracycline, an unexpected abrupt febrile response often occurred within 8 to 12 hours after the initial dose of the antibiotic. These adverse reactions could be abolished or ameliorated by administering smaller initial doses of the antibiotic. Such effects suggested to the observers that, as a result of the antibrucella activity of the chlortetracycline, brucella cells were killed, thus liberating some cellular component which then produced the febrile response. This component was presumably the endotoxin of the cell.

That brucellae contain endotoxin has been amply demonstrated by several observers (2–5). This endotoxin, just as that from many other gram-negative microorganisms, consists of lipopolysaccharide-protein complexes located on the surface of the bacterium. It represents not only a toxic component of the cell but also the somatic or "O" antigen. The properties of brucella endotoxin have been extensively studied in the experimental animal (6–8) and have been found to be quite similar to those of endotoxins from other organisms (9). Of particular interest are the observations (3, 10) that endotoxins extracted from various strains of the three brucella species have identical toxic and immunologic actions, regardless of the capacity of the individual strain to establish infection. In man, the injection of endotoxins from various enteric microorganisms causes fever, headache, chilliness, myalgia, emesis, leukopenia, alterations in carbohydrate metabolism, and vasomotor disturbances which may culminate in shock if sufficient endotoxin is given (11, 12). Morgan and Neva (13, 14) observed such reactions in subjects given typhoid and paratyphoid endotoxins. Because of the similarity of these reactions to the symptoms of patients with typhoid and paratyphoid fever, it was postulated that endotoxin played a significant role in the pathogenesis of illness in these diseases.

The purpose of the present investigation was to study the role of endotoxin in the pathogenesis of illness in human brucellosis. This was done by observing the reactions of human subjects to the intravenous injection of brucella endotoxin. Such studies have not been previously reported.

MATERIAL AND METHODS

Endotoxin was extracted from *Br. melitensis* by a modified Boivin procedure (15). In all human tests, the same lot of endotoxin was used. The preparation was a saline suspension containing 0.5 per cent phenol and was stored at 4° C. over a period of three years. No deterioration or contamination occurred. In humans, endotoxin was given intravenously; the response was measured by observing the systemic reactions of each individual and by recording body temperature orally at one or two hour intervals, beginning one hour prior to challenge and continuing for at least eight hours after challenge or thereafter until the temperature returned to the base line. All patients tested had normal temperatures for at least 48 hours previously; no salicylates were administered during the test period. Fever, as a response to endotoxin, was defined as temperature over 100° F. In order to compare the individual reactions objectively, the febrile response was quantitated in terms of fever units (14), a value obtained by multiplying the degrees of fever above 100° F. times the duration of fever in hours. In practice, this was done by plotting each individual temperature response on graph paper con-

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TABLE I

Classification of patients given brucella endotoxin

Classification of groups	Number of patients	Brucella skin test	Serum brucella agglutinins	History of active disease
Control	16	negative	absent	absent
Inactive disease	12	positive	$\leq 1:80$	absent
Active disease	13	positive	$>1:80$	present

taining 400 small squares per square inch, using the following scales: along the abscissa 1 inch equals 12 hours, and along the ordinate 1 inch equals 2° F. Each individual square thus represented 0.06 fever unit. The total number of fever units was determined by counting the number of small squares enclosed within the area of the temperature above 100° F.

The subjects given endotoxin were all hospitalized patients. Three categories of subjects were chosen, the classification of each patient being based upon the following: history of an active disease compatible with brucellosis; serum brucella agglutinin titers; cultures for brucellae; and intradermal reactions to brucella skin test antigens [either the nucleoprotein brucellergin or the detergent-extracted protein antigen of Benedict and Elberg [2] (16)]. The essential distinguishing characteristics of each group are presented in Table I. The 13 individuals designated as having active brucellosis consisted predominantly of patients hospitalized for treatment of acute or subacute brucellosis. Included also in this group were three subjects who had apparently recovered completely from a previous acute brucellosis and two individuals with chronic brucellosis, characterized by a significant exposure to brucellae associated with recurrent febrile episodes and high serum brucella agglutinins at some stage of their illness. All of the patients in this category had demonstrated either positive cultures for brucellae or significant agglutinins ($>1:160$) and possessed dermal hypersensitivity for brucella antigens. For comparison, a second group was composed of 12 patients who demonstrated brucella dermal hypersensitivity but gave no history suggesting active brucellosis (either past or present) and had low or absent serum agglutinins. These were selected as individuals with an apparently inactive or subclinical brucella infection. A final control group consisted of 16 patients with no evidence of brucella infection at any time; they all had absent brucella agglutinins and no dermal reactions to brucella antigen. The last two groups were patients initially hospitalized with various illnesses but were apparently similar save for the dermal hypersensitivity; none of the patients were seriously ill at any time.

To investigate the development of resistance to endotoxin activity during human brucellosis, the capacity of serum from patients with brucellosis to protect against the lethality of endotoxin was measured in mice. Male ABC mice, weighing approximately 20 grams each, were used; they were housed 5 to 10 per cage in an air-conditioned room, with free access to tap water and Fox Chow. All injections were given intraperitoneally. The amount of endotoxin used for challenge was routinely a 1.5 LD_{50} amount; by weight this varied from 0.9 to 1.2 mg., depending on the potency of the individual preparation. When the protective effect of human serum was tested, the serum and endotoxin were mixed together and then injected.

Serum precipitins for brucella endotoxin were determined by a capillary tube technique. Endotoxin, in a concentration of 1 mg. per ml. of saline, was drawn into a capillary tube, after which an equal volume of serum was drawn in. The tube was placed in a strip of plasticene embedded in a wooden block, then incubated initially at 37° C. for 1 hour, and finally kept at 4° C. for approximately 18 hours. The majority of sera tested had been stored in the frozen state for varying intervals up to five years.

EXPERIMENTAL RESULTS

A. Occurrence of endotoxin antibodies in brucellosis

Serologic evidence that exposure to the endotoxic component of the brucella cell occurs during the course of human brucellosis was sought by performing precipitin reactions for endotoxin and also by determining the protective capacity of such sera against the lethality of brucella endotoxin for mice.

Precipitins. The sera of 43 patients with active brucellosis were tested for precipitins, a total of 49 sera being tested. Of the 43 patients, 19 gave positive precipitin reactions at some stage of their illness. In Table II, the precipitin reactions are correlated with the duration of clinical disease, a time interval measured from the onset of symptoms. It is seen that precipitins may appear quite early, persist some 6 months, and then begin to decline by 9 to 12 months. When the precipitins were compared with the brucella agglutinin titers

TABLE II

Correlation of precipitin reaction with duration of brucellosis

Duration of disease	Number sera tested	Number sera positive	Per cent positive
1–2 weeks	17	7	41%
3–4 weeks	7	3	43%
2–4 months	8	5	63%
5–6 months	6	3	50%
9–12 months	4	1	25%
2–12 years	6	0	0%

[2] Kindly supplied by Dr. S. S. Elberg.

TABLE III

Correlation of serum precipitin reactions with serum agglutinin titers in human brucellosis

Serum agglutinin titer	Serum precipitin reaction	
	Number positive	Number negative
0	0	2
1:80	0	1
1:320	1	3
1:640	3	9
1:1,280	3	7
1:2,560	5	4
1:5,120	6	1
1:10,240	2	1
1:20,480	1	0

of the same sera (see Table III), it was observed that of those sera with an agglutinin titer of 1:2,560 or greater, 70 per cent gave positive precipitin reactions, while in contrast only 24 per cent of those sera with lower agglutinin titers had precipitins.

Passive protection of mice against endotoxin. Sera from 19 patients with brucellosis were tested for their capacity to protect mice against the lethality of brucella endotoxin. Preliminary tests with serum from Patient M. W. indicated that 0.4 ml. serum protected mice, whereas 0.2 ml. did not. Accordingly, the protective activity of individual sera was tested by challenging mice with a mixture of a lethal dose of endotoxin and either 0.3 or 0.4 ml. serum, depending upon the amount available. Sera from five normal individuals were similarly tested, while a control group of mice received only endotoxin. The results are given in Table IV. Since the number of animals for each serum was so small, only those sera protecting at least four of five mice were designated as offering protection. While none of the control normal sera protected mice, nine of the brucellosis sera (or 47 per cent) did give protection. All of these nine sera were found to contain precipitins for the endotoxin; however, three of five sera giving no protection also contained precipitins. Of the 11 sera with agglutinin titers of 1:1,280 or less, only 3 (27 per cent) afforded protection; in contrast, of the 7 sera with agglutinin titers of 1:2,560 or higher, 5 (72 per cent) gave protection.

B. Reactions to brucella endotoxin in humans

The responses to intravenous injections of brucella endotoxin were observed in 41 individuals

TABLE IV

Effect of human immune serum on brucella endotoxin lethality in mice

Patient	Duration of disease	Precipitin reaction	Agglutinin titer	Amount serum given	Endotoxin mortality*
W. L.	3 weeks	+	1:320	0.4 ml.	5/5
J. R.	3 weeks	+++	1:2,560	0.4 ml.	2/4
J. D.	3 weeks	+++	1:2,560	0.3 ml.	3/5
A. S.	5 weeks	+	1:5,120	0.4 ml.	1/5
F. S.	6 weeks	+	1:10,240	0.4 ml.	0/5
J. H.	6 weeks		1:1,280	0.4 ml.	1/5
B. S.	2 months		1:640	0.4 ml.	5/5
A. B.	2 months	0	1:1,280	0.3 ml.	5/5
J. A.	2 months	+	1:1,280	0.4 ml.	2/5
S. D.	3 months	+	1:640	0.4 ml.	2/5
R. P.	4 months	+	1:1,280	0.3 ml.	1/5
L. S.	6 months	++	1:10,240	0.4 ml.	0/5
C. K.	6 months	+++	1:5,120	0.4 ml.	0/5
P. A.	6 months	++	1:640	0.3 ml.	0/5
H. H.	14 months		1:160	0.3 ml.	3/5
K. E.	3 years		1:320	0.4 ml.	2/5
M. W.	1 month	++	1:5,120	0.4 ml.	0/5
R. D.				0.4 ml.	2/10
F. H.	6 months	0	1:320	0.4 ml.	5/5
Control 1		0	0	0.3 ml.	4/5
Control 2		0	0	0.4 ml.	3/5
Control 3		0	0	0.4 ml.	7/10
Control 4		0	0	0.4 ml.	10/10
Control 5				0.4 ml.	5/5
Saline				0.4 ml.	33/37

* Number dying/number challenged.

who were divided into three groups as described under the section Material and Methods. In order to determine if differences in suceptibility to endotoxin existed among the three groups, varying amounts of endotoxin were given to the individuals in each group. The data showing the responses in each group are detailed in Table V for the active group, in Table VI for the apparent inactive group, and in Table VII for the control group.

Systemic response to endotoxin. The systemic response to the intravenous injection of endotoxin characteristically included headache, chilliness or rigors, malaise, and myalgia—all prominent symptoms in brucellosis. Since these subjective complaints could not be measured in any precise quantitative fashion, the magnitude of the response was estimated as minimal, moderate or marked. The reactions in each group have been summarized in Table VIII. In the control group, 15 individuals received either 50 or 100 γ endotoxin and experienced no or minimal reactions save for one person with a moderate reaction. The remaining control patient suffered a moderate reaction when given 250 γ endotoxin. Patients with inactive or subclinical infection were given amounts of endotoxin ranging from 1 to 100 γ; 7 of the 12 gave no or minimal reactions, while 5 gave moderate or marked responses. The responses of the group with active brucellosis were

TABLE V

Response to brucella endotoxin in patients with active brucellosis

Patient Age	Sex	Stage of brucellosis	Duration of illness	Skin test	Agglutinins	Precipitins	Amount of endotoxin	Febrile reaction: Time of onset	Febrile reaction: Duration	Febrile reaction: Fever units	Systemic reaction
M. O. 19	M	Acute	5 months	++	1:5,120	++	10 γ	5 hrs.	11 hrs.	6.8	Marked
E. S. 31	M	Chronic	6 years	++	1:320	0	10 γ			0	None
R. M. 45	M	Healed	2 years	++	0	0	10 γ	10 hrs.	15 hrs.	5.3	Moderate
W. J. 28	M	Acute	6 months	++	1:640	0	25 γ	2 hrs.	45 hrs.	77.6	Marked
F. S. 22	M	Acute	2 months	+	1:2,560	+	25 γ	1.8 hrs.	15 hrs.	5.9	Moderate
S. D. 38	M	Acute	3 months	++	1:640	+	25 γ	4 hrs.	30 hrs.	61.5	Marked
J. H. 25	M	Acute	7 weeks	+	1:10,240		25 γ	3.6 hrs.	6 hrs.	11.2	Moderate
D. L. 54	M	Subacute	8 months	++	1:320	0	25 γ	12 hrs.*	10 hrs.*	13.4*	Moderate*
L. Ba. 46	M	Healed	6 years	++	0	0	25 γ	7 hrs.*	30 hrs.*	36.5*	Marked*
J. R. 36	M	Acute	1 month	+++	1:5,120	+++	50 γ	2 hrs.	33 hrs.*	13.0*	Marked*
S. N. 27	M	Healed	4 years	−	1:80	0	250 γ	0.5 hrs.	42 hrs.*	61.7*	Marked*
J. A. 26	M	Acute	2 months	+++	1:1,280	+	1 γ (Day 1) 5 γ (Day 3)	 2 hrs.	 5 hrs.	0 0.9	None Minimal
S. T. 59	F	Chronic	12 years	++	0	0	5 γ (Day 1) 10 γ (Day 2) 20 γ (Day 5) 30 γ (Day 9)	 5 hrs. 2.5 hrs. 10.8 hrs.	 12 hrs. 12 hrs. 1.2 hrs.	0 3.2 10.3 0.2	None Moderate Moderate None

* Given steroid treatment.

TABLE VI

Response to brucella endotoxin in patients with inactive brucellosis

Patient Age	Sex	Clinical diagnosis	Skin test	Agglutinins	Precipitins	Amount of endotoxin	Febrile reaction: Time of onset	Febrile reaction: Duration	Febrile reaction: Fever units	Systemic reaction
P. B. 51	M	No disease	++++	1:80	0	1 γ			0	Moderate
T. R. 22	F	Rheumatic fever	++++	1:80	+	1 γ	10 hrs.	30 hrs.	60	Marked
R. T. 56	M	Polyneuritis	+++	1:40		5 γ			0	None
M. T. 44	F	Arthritis	+	1:20	0	10 γ	1 hr.	2 hrs.	1.4	Moderate
A. A. 54	F	Tuberculosis	+++	0	+	10 γ	6 hrs.	23 hrs.	63.8	Moderate
M. H. 46	F	Renal abscess	+	0	0	50 γ	1.5 hrs.	2 hrs.	2	Minimal
W. K. 73	M	Anemia	+	1:40	0	50 γ	12 hrs.	14 hrs.	9.2	None
H. U. 43	F	CA rectum	+	0	0	50 γ			0	None
L. B. 67	M	CA esophagus	+			50 γ			0	None
R. A. 31	M	No disease	+	1:40		50 γ			0	Minimal
V. H. 54	M	Duodenal ulcer	+	1:40	0	100 γ	2 hrs.	24 hrs.	21	Marked
C. K. 41	F	Neurosis	++	1:20	0	10 γ (Day 1)	15 hrs.	8 hrs.	1.5	Moderate
						20 γ (Day 4)	1.5 hrs.	9 hrs.	8.9	Moderate
						40 γ (Day 8)	4 hrs.	4 hrs.	1.6	Minimal

TABLE VII

Response to brucella endotoxin in control patients

Patient	Age	Sex	Clinical diagnosis	Amount of endotoxin	Reaction to endotoxin: Fever units	Reaction to endotoxin: Systemic
G. S.	56	M	Duodenal ulcer	50 γ	0	None
E. M.	39	M	Pneumonia	50 γ	0	Minimal
L. I.	70	F	CA breast	50 γ	0.3	Minimal
F. B.	64	M	Leukemia	50 γ	0	Moderate
J. O.	36	F	Pneumonia	50 γ	0	Minimal
O. H.	62	M	Arthritis	50 γ	0	None
P. G.	47	M	Diabetes; cellulitis	50 γ	0	None
W. S.	55	M	Parkinsonism	50 γ	0	None
C. A.	21	F	Hepatitis	100 γ	0	None
G. Ko.	49	M	Acute alcoholism	100 γ	0	Minimal
E. H.	83	M	Duodenal ulcer	100 γ	0	None
J. M.	76	M	Pulmonary infarct	100 γ	0	None
G. Ke.	40	M	Thrombophlebitis	100 γ	0	None
J. D.	56	F	Duodenal ulcer	100 γ	0	None
M. F.	61	M	Asthma	100 γ	0	None
H. M.	53	M	Sarcoidosis	250 γ (Day 1)	10.9	Moderate
				250 γ (Day 2)	12.3	Moderate

TABLE VIII

Summary of reactions to endotoxin in humans

Classification of groups	Number of patients with symptoms				Number of patients with fever			
	None	Minimal	Moderate	Severe	None	Minimal ≤2 F.U.	Moderate 5–15 F.U.	Severe >20 F.U.
Controls	10	4	2	0	15	0	1	0
Inactive disease	4	3	3	2	5	2	2	3
Active disease	1	1	5	6	1	1	7	4

almost uniformly more severe than the other groups. Twelve of these individuals were challenged with 1 to 50 γ endotoxin, while the remaining patient received 250 γ. Only 2 patients experienced no or minimal reactions, while in contrast 11 gave moderate or marked reactions. These results show clearly that those individuals with active brucellosis possess an increased susceptibility to the systemic effects of endotoxin.

Febrile response to endotoxin. The febrile reactions in the three groups have been summarized in Table VIII. Those control individuals receiving 50 or 100 γ had no febrile reactions; however, when challenged with 250 γ, fever did result. The subjects with inactive infection were less resistant; five of them experienced significant febrile responses to smaller amounts of endotoxin. In contrast, the patients who had active brucellosis were much more susceptible, since all but 2 of the 13 individuals suffered prominent febrile reactions.

Responses characteristic of each group are illustrated in Figure 1, which presents the reactions of three patients to 50 γ endotoxin. G. S., a control patient, had no febrile or systemic reaction. M. H., an individual whose only evidence of brucella infection was a positive skin test, experienced only a slight but distinct response. In contrast, Patient J. R., who was convalescing from acute brucellosis, suffered a marked febrile and systemic response.

This variation in susceptibility is shown in greater detail by correlating the individual febrile responses with the amount of endotoxin given (see Figure 2). For the sake of clarity, the responses of the control group have been omitted from the figure; if shown, the reactions to 50 or 100 γ would all be clustered at 0 fever units. The increased susceptibility to endotoxin in the other two groups is indicated by the significant febrile responses to 10, 25, or 50 γ endotoxin, amounts without effect in controls. An analysis of Figure 2 shows that the responses fell into three broad categories: minimal reactions below 2 fever units, moderate reactions of 5 to 15 fever units, and severe reactions greater than 20 units. Individuals with minimal reactions were predominantly those with inactive infections. In contrast, moderate reactors were predominantly patients with active brucellosis. Severe reactions occurred equally in both groups. This apparent

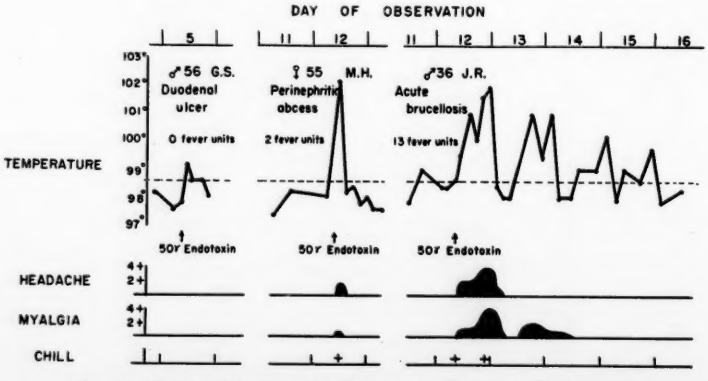

FIG. 1. TYPICAL RESPONSES OF HUMANS TO BRUCELLA ENDOTOXIN

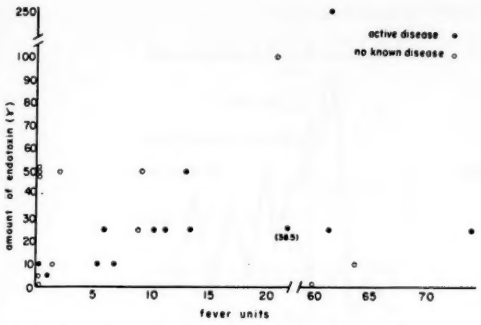

FIG. 2. FEBRILE RESPONSE TO BRUCELLA ENDOTOXIN

discrepancy may best be explained by the observation that the two patients with inactive infection experiencing severe reactions both possessed marked dermal hypersensitivity; this point will be further discussed shortly.

It was observed that a latent period occurred between the injection of endotoxin and the onset of the febrile response. The duration of this latent period, the duration of the entire febrile reaction, and the number of fever units were compared in the group with active disease and in the subclinically infected group. Table IX presents a summary of data abstracted from Tables V and VI, giving the mean value with standard deviation and ranges. Those patients with active brucellosis had a distinctly shorter latent period and a somewhat more prolonged duration of the febrile response. No correlation was found between the amount of endotoxin given and the duration of either the latent period or the febrile response.

Factors influencing the intensity of the response to endotoxin. When the responses of the various individuals were analyzed, it was seen that two factors seemed to influence greatly the individual reaction to endotoxin. The first, as stressed in the preceding discussion, was the occurrence of an active infection. The other important factor was the degree of dermal hypersensitivity to brucella skin test antigens.

The increased susceptibility in patients who had active disease was apparent even in individuals possessing equal dermal hypersensitivity, as illustrated in Figure 3, which contrasts the responses to 25 γ endotoxin in two patients with skin tests of moderate (2 plus) intensity. Patient W. J., who was recovering from acute brucellosis, experienced a severe reaction; on the other hand, Patient C. K., with an inapparent infection, had only a moderate reaction. It was also observed that the increased susceptibility persisted long after apparent complete recovery, for intervals of two to six years (see Table V, Patients R. M., S. N., and L. Ba.).

Correlation of the febrile responses to endotoxin with the degree of dermal hypersensitivity (Table X) indicated that in several instances those patients with marked dermal hypersensitvity gave much more severe reactions to the same amount of endotoxin than did patients with a low degree of dermal hypersensitivity. An example of this is presented in Figure 4, which shows the reactions of two patients with inactive infection to the injection of 10 γ endotoxin. Patient M. T., with only a 1 plus skin test, suffered little reaction, but Patient A. A., who had a 3 plus skin test, experienced a severe reaction.

Development of tolerance to endotoxin. Six individuals were given two or more injections of endotoxin at various intervals. As detailed in Table XI, resistance to the febrile and systemic actions of endotoxin developed in three subjects. This tolerance was not demonstrated in those patients receiving a second injection within five days but was observed in the three individuals given endotoxin six days or more after the initial challenge.

TABLE IX

Comparison of febrile responses to endotoxin in humans with active and inactive brucellosis

Classification of group	Time of onset of reaction	Duration of reaction	Fever units
Active disease	3.3 ± 0.9 hrs. (0.5–10 hrs.)	20.5 ± 3.7 hrs. (5–45 hrs.)	14.4 ± 7.2 (0–78)
Inactive disease	6.1 ± 1.9 hrs. (1–15 hrs.)	14.0 ± 4.0 hrs. (2–30 hrs.)	12.9 ± 5.7 (0–64)

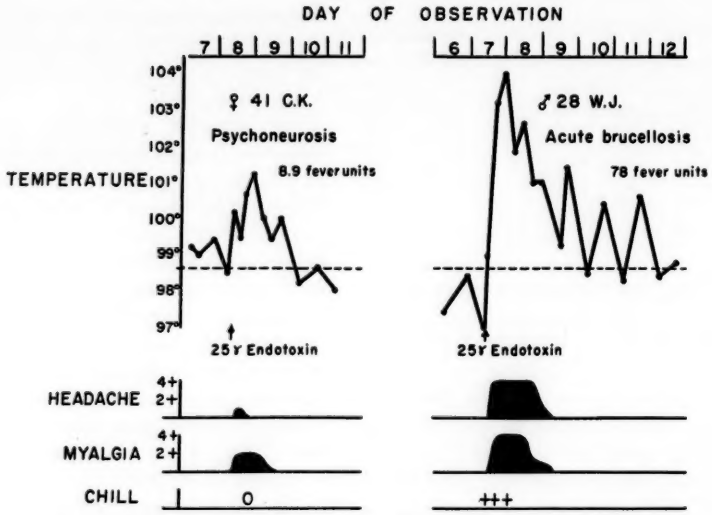

FIG. 3. EFFECT OF ACTIVITY OF DISEASE ON REACTION TO ENDOTOXIN

It may be noted that tolerance appeared not only after repeated injections of endotoxin (Patients S. T. and C. K.) but also after a single injection (Patient J. H.).

Effect of adrenocorticosteroids and adrenocorticotropic hormone on response to endotoxin. Five patients with active brucellosis were given either hydrocortisone or adrenocorticotropic hor-

TABLE X

Correlation of dermal hypersensitivity and febrile response to endotoxin in human brucellosis

Amount of endotoxin	Individual reactions to endotoxin (fever units)						
	+		++		+++		++++
	Active	Inactive	Active	Inactive	Active	Inactive	Inactive
1 γ							0 60
5 γ			0		0.9	0	
10 γ		1.4	0 3.2 5.3 6.8	1.5		63.8	
25 γ	11.2 5.9		10.3 13.4* 36.5* 61.5 77.6	8.9			
50 γ		0 0 0 2 9.2			13*		
100 γ		21					
250 γ			61.7*				

* Patient given steroid therapy.

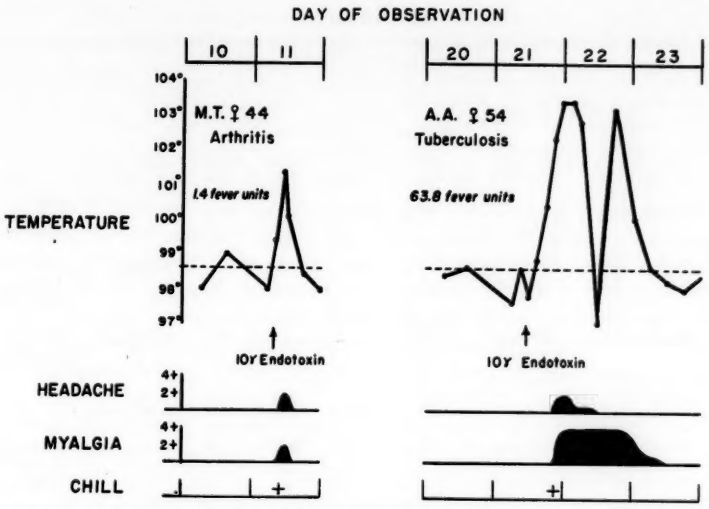

FIG. 4. EFFECT OF DERMAL HYPERSENSITIVITY ON REACTION TO ENDOTOXIN

mone (ACTH) in conjunction with challenge with endotoxin. The effects on the responses to endotoxin are summarized in Table XII.

In Patient F. S., the intravenous injection of 100 mg. of hydrocortisone over a six hour period prevented any adverse reaction to challenge with 25 γ endotoxin, a dose which was toxic for this subject, as demonstrated by a distinct febrile and systemic response when given two days later without hydrocortisone. While the similar administration of 100 mg. of hydrocortisone did not prevent the appearance of a moderate reaction to 25 γ endotoxin in Patient D. L., it did delay the onset of the reaction until six hours after completion of therapy with hydrocortisone. In contrast to these two patients, the simultaneous injection of 100 mg. of hydrocortisone hemisuccinate with 25 γ endotoxin did not prevent a marked reaction in Patient L. B. However, when a second injection of 100 mg. of hydrocortisone hemisuccinate was given at the height of the response, a prompt fall in fever and a diminution of the systemic response resulted and persisted for 10 hours, after which a secondary relapse occurred.

TABLE XI

Development of tolerance to endotoxin in humans

Patient	Classification of disease	Initial injection		Subsequent injections		
		Amount of endotoxin	Fever units	Time after initial dose	Amount of endotoxin	Fever units
J. A.	Active	1 γ	0	Day 2	5 γ	0.9
F. S.	Active	25 γ*	0	Day 3	25 γ	5.9
J. H.	Active	25 γ	11.2	Day 6	25 γ	0.7
S. T.	Active	5 γ	0	Day 2	10 γ	3.2
				Day 5	20 γ	10.3
				Day 9	30 γ	0.2
C. K.	Inactive	10 γ	1.5	Day 4	25 γ	8.9
				Day 8	40 γ	1.4
H. M.	Control	250 γ	10.9	Day 2	250 γ	12.3

* Given hydrocortisone.

TABLE XII

Effect of adrenocorticotropic hormone and steroid therapy on reaction to endotoxin in human brucellosis

Patient	Stage of disease	ACTH or steroid therapy: Time (in relation to endotoxin)	ACTH or steroid therapy: Amount and type of therapy	Amount of endotoxin	Reaction to endotoxin: Febrile response: Onset	Febrile response: Duration	Febrile response: Fever units	Reaction to endotoxin: Systemic response	Effect of therapy
S. N.	Healed	8 hrs. after	15 U (units) ACTH I.V.	250 γ	0.5 hr.	42 hrs.	61.7	Marked	Fall in fever by 6 hrs.; recurrence 12 hrs. later.
J. R.	Acute	14 hrs. after	20 U (units) ACTH I.V.	50 γ	2 hrs.	33 hrs.	13	Marked	Afebrile and asymptomatic by 3 hrs.; recurrence after 12 hrs.
F. S.	Acute Day 1	Simultaneous	100 mg. hydrocortisone I.V.	25 γ			0	Minimal	Excellent suppression of reaction.
	Day 3		None	25 γ	2 hrs.	15 hrs.	5.9	Moderate	
D. L.	Subacute	Simultaneous	100 mg. hydrocortisone I.V.	25 γ	12 hrs.	10 hrs.	13.4	Moderate	Probable delay in onset of reaction; weaker systemic response.
L. D.	Healed	Simultaneous and 8 hrs. later	100 mg. hydrocortisone I.V. ×2	25 γ	7 hrs.	30 hrs.	36.5	Marked	? delay in onset; fall in fever by 4 hrs. after 2nd dose, recurrence at 10 hrs.

ACTH was administered to two individuals in an attempt to alleviate severe responses to endotoxin. When 20 mg. of ACTH was given over a six hour period to Patient J. R., suffering a marked toxic reaction to 50 γ endotoxin, a dramatic decline in fever and toxemia resulted, so that within three hours the patient was asymptomatic. However, 12 hours later fever and myalgia of moderate intensity recurred, then subsided spontaneously. The response of Patient S. N. was less clear-cut. During a severe reaction to the relatively large dose of 250 γ endotoxin, 15 mg. of ACTH was injected over a three hour interval. The temperature fell slowly over the next six hours and remained normal for 12 hours; the fever and systemic response then recurred and persisted an additional 36 hours.

Immunologic response to endotoxin. Whenever possible, serum precipitins for endotoxin and agglutinins for intact brucella cells were tested both before and after injection of endotoxin. Since the majority of patients were seen only during hospitalization, systematic testing at intervals was not feasible. The results, therefore, do not represent a maximal immunologic response. In 12 patients with negative precipitin reactions prior to receiving endotoxin, 7 gave positive reactions when tested at later dates. Similarly, 9 of 10 patients with initial absent or low (1:80 or less) agglutinin titers developed significant titers (1:320 to 1:5,120) subsequently. The data were insufficient to permit any detailed conclusions regarding either time of appearance or duration of these antibodies, but in one individual they developed within one week and persisted as long as two years.

DISCUSSION

The reactions of humans to brucella endotoxin have not been studied in this fashion previously. Pirosky and Molinelli (17) used a Boivin-type of endotoxin extracted from *Brucella suis* as a skin-test antigen in studying individuals from the littoral region of Argentina, an endemic area for brucellosis. They found that those individuals with active brucellosis or those recovered from active disease (6 months to 22 years previously) uniformly gave positive reactions of the delayed type, whereas controls showed no reactions. Some of the subjects with positive intradermal reactions also experienced febrile responses to endotoxin.

In their study of the effects of endotoxin upon patients convalescing from typhoid and paratyphoid fevers, Neva and Morgan (14) gave in-

creasing doses of typhoid and shigella endotoxin over four consecutive days in order to investigate the development of tolerance. Nevertheless, from their data it can be determined that these patients with salmonella infections were more resistant than controls and gave less febrile or systemic reactions to the initial injection of either typhoid or shigella endotoxin. Of interest was the observation that patients with various other infectious diseases all reacted in the same fashion as controls. Heyman and Beeson (18) measured the febrile response to a single injection of dead intact *S. typhosa* cells in patients with a wide variety of diseases; this pyrogenic response most probably represented a reaction to the endotoxin of the cells. Only two convalescent typhoid patients were included but both gave increased febrile responses as compared with controls. The only other subjects demonstrating a consistent increased susceptibility were patients with hepatic cirrhosis. Conversely, resistance to the pyrogenic effect was found in a group of patients undergoing malaria therapy for neurosyphilis. It was speculated that this resistance might be due to a hyperactivity of the reticuloendothelial system in response to the malaria. The observations of Neva and Morgan give somewhat conflicting results from those of Heyman and Beeson as concerns susceptibility to endotoxin in typhoid fever. The increased resistance described by Neva and Morgan contrasts sharply with the susceptibility to endotoxin found in the patients with brucellosis.

The pattern of the febrile responses to brucella endotoxin differed significantly from those previously described in individuals given typhoid and shigella endotoxins (14) or typhoid vaccine (18). With these latter agents, fever uniformly appeared within 30 to 60 minutes and was rarely prolonged beyond six to eight hours. In contrast, with brucella endotoxin there was usually a delay of several hours before fever appeared, and the reaction was sustained more than 10 hours in almost all of the patients. The mechanism of this difference is not apparent. The preparation of brucella endotoxin used was a colloidal suspension; it is conceivable that the delayed reactions reflected simply a difference in physical characteristics of this material from the endotoxins employed by other investigators. On the other hand, it is also possible that the sustained reactions represented a specific reaction of these subjects to brucella endotoxin and therefore differed from the comparatively short-lived nonspecific responses of humans to bacterial endotoxins in general.

While the exact contribution to the symptomatology of brucellosis of this demonstrated sensitivity to brucella endotoxin is conjectural, it is significant that many of the patients with active brucellosis stated that the reaction to brucella endotoxin reproduced almost completely the complaints experienced during the acute phase of their illness. Accordingly, it would appear quite likely that many of the overt manifestations of infection with brucellae could well represent the reaction of the infected host to the endotoxin of the organism, as Morgan has postulated for typhoid fever. The accentuation of susceptibility to endotoxin in individuals who have had active disease and in those with prominent dermal hypersensitivity suggests that this is a manifestation of generalized hypersensitivity to the endotoxin. For further support of such a concept, it would be desirable to determine the reactions of subjects with brucellosis to endotoxins extracted from other microorganisms, since it has been amply demonstrated that all bacterial endotoxins possess similar toxic actions for normal humans. Pending the results of such observations, it is more accurate to employ the term "increased susceptibility" rather than hypersensitivity.

These studies also indicate that exposure of the human host to endotoxin may result in the formation of protective mechanisms against endotoxin. In support of this concept is the demonstration of passive protection for mice against lethal doses of endotoxin with the serum of patients with brucellosis. That this possibly occurs through an immunologic mechanism is suggested by its correlation with serum precipitins and agglutinins. This is in contrast to the lack of correlation between the amount of serum antibodies and the degree of reaction to endotoxin, as also noted by Morgan and Neva (13, 14). The capacity of these patients to develop tolerance to endotoxin following one or more injections also represents in effect a protective measure. This tolerance developed in a fashion similar to that described for typhoid endotoxin by Morgan, who found that tolerance appeared after 8 to 10 days of repeated injections. Of particular interest in this

regard was the observation that resistance to brucella endotoxin developed in one individual following only one injection of endotoxin. In the mouse, evidence of the protective capacity of endotoxin has been obtained (19). Following injections of brucella endotoxin, resistance to endotoxin lethality develops and persists for months. In addition, protection against challenge with viable brucellae is conferred.

The beneficial effect of hydrocortisone or ACTH in ameliorating or preventing the toxicity of brucella endotoxin in humans conforms with the protective action of the adrenocorticosteroids against endotoxin lethality in mice (10, 20). It also suggests that the beneficial action of therapy of acute brucellosis with ACTH (21) or steroids (22) may result in part through a suppression of endotoxin activity. In addition, these findings add further evidence to repeated observations that steroid therapy is helpful in the treatment of serious infections due to gram-negative microorganisms (23) in which endotoxin probably contributes to illness.

From these results in humans, coupled with observations in the mouse, the role of endotoxin in the pathogenesis of brucellosis may be envisioned thusly. Upon invasion of the host by brucellae, host defense mechanisms result in disruption of the bacterial cells with liberation of endotoxin. If a sufficient quantity floods the tissues, the host responds with a febrile and systemic response. Such a reaction may occur in any individual provided sufficient endotoxin is liberated. However, it would appear that during infection a state of increased susceptibility develops and renders the host more sensitive to the actions of endotoxin, so that severe reactions may occur with amounts of endotoxin that are harmless to noninfected individuals. While this altered reactivity is significant in the causation of illness in brucellosis, other factors as yet unelucidated may be equally important. In contrast to this undesirable role, endotoxin also has a protective role, as shown by the development of serum antibodies, the development of resistance to endotoxin and the appearance of antibacterial resistance to infection following injections of endotoxin. These conflicting dual roles again emphasize the complexity of host reactions in infectious diseases.

SUMMARY

1. Sera from patients with brucellosis were found to contain precipitins for brucella endotoxin and to confer passive protection against the lethality of brucella endotoxin for mice.

2. The injection of sufficient quantities of brucella endotoxin into humans caused fever, chills, sweating, myalgia, headache, and malaise. Individuals who had been infected by brucellae were much more susceptible to these effects than noninfected control subjects.

3. The degree of reaction to brucella endotoxin was more severe in those individuals who had experienced active brucellosis (either present or in the past) and in those with more intense intradermal reactions to brucella skin test antigens.

4. Resistance to the toxicity of brucella endotoxin developed after repeated injections of endotoxin.

5. Hydrocortisone or adrenocorticotropic hormone suppressed or ameliorated reactions to brucella endotoxin in five patients.

6. Injections of brucella endotoxin into humans caused the formation of serum precipitins for the endotoxin and serum agglutinins for brucella cells.

7. It is suggested that brucella endotoxin contributes significantly to the pathogenesis of illness in brucellosis, particularly in the infected host who has been made more susceptible to its toxicity.

REFERENCES

1. Spink, W. W., Braude, A. I., Castaneda, M. R., and Goytia, R. S. Aureomycin therapy in human brucellosis due to Brucella melitensis. J. Amer. med. Ass. 1948, **138**, 1145.
2. Miles, A. A., and Pirie, N. W. The properties of antigenic preparations from Brucella melitensis. I. Chemical and physical properties of bacterial fractions. Brit. J. exp. Path. 1939, **20**, 83.
3. Lisbonne, H., and Monnier, P. Quelques propriétés de l'antigène complet (Boivin) de Brucella melitensis. C. R. Soc. Biol. (Paris) 1936, **123**, 1114.
4. Damboviceanu, A., Barber, C., Pop, A., and Marinov, I. Ontigène complet des brucella. Caractères chimiques. C. R. Soc. Biol. (Paris) 1938, **127**, 736.
5. Pennell, R. B. The chemistry of *Brucella* organisms *in* Brucellosis. Washington, D. C., American Ass. for the Advancement of Science, 1950, p. 37.
6. Miles, A. A., and Pirie, N. W. The properties of antigenic preparations from Brucella melitensis.

III. The biological properties of the antigen and the products of gentle hydrolysis. Brit. J. exp. Path. 1939, **20**, 278.

7. Paterson, J. S., Pirie, N. W., and Stableforth, A. W. Protective antigens isolated from Br. abortus. Brit. J. exp. Path. 1947, **28**, 223.
8. Pop, A., Damboviceanu, A., Barber, C., and Marinov, I. Antigène complet des brucella. Propriétés biologiques. C. R. Soc. Biol. (Paris) 1938, **127**, 733.
9. Van Heyningen, W. E. Bacterial Toxins. Oxford, Blackwell Scientific Publications, 1950.
10. Spink, W. W., and Anderson, D. Experimental studies on the significance of endotoxin in the pathogenesis of brucellosis. J. clin. Invest. 1954, **33**, 540.
11. Bennett, I. L., Jr., and Beeson, P. B. The properties and biologic effects of bacterial pyrogens. Medicine 1950, **29**, 365.
12. Thomas, L. The physiological disturbances produced by endotoxins. Ann. Rev. Physiol. 1954, **16**, 467.
13. Morgan, H. R. Resistance to the action of the endotoxins of enteric bacilli in man. J. clin. Invest. 1948, **27**, 706.
14. Neva, F. A., and Morgan, H. R. Tolerance to the action of endotoxins of enteric bacilli in patients convalescent from typhoid and paratyphoid fevers. J. Lab. clin. Med. 1950, **35**, 911.
15. Halberg, F., and Spink, W. W. The influence of Brucella somatic antigen (endotoxin) upon the temperature rhythm of intact mice. Lab. Invest. 1956, **5**, 283.
16. Benedict, A. A., and Elberg, S. S. Cutaneous hypersensitivity in brucellosis. I. Characterization of an antigen for detection of cutaneous hypersensitivity in brucellosis. J. Immunol. 1953, **70**, 152.
17. Pirosky, I., and Molinelli, E. A. Estudios sobre alergia brucelosa. I. Empleo del complejo glucidolipido de Brucella en la investigacion alergica del hombre. Rev. Med. Cienc. afin. (Méx.) 1944, **6**, 850.
18. Heyman, A., and Beeson, P. B. Influence of various disease states upon the febrile response to intravenous injection of typhoid bacterial pyrogen. J. Lab. clin. Med. 1949, **34**, 1400.
19. Abernathy, R. S. Homologous and heterologous resistance in mice given bacterial endotoxins. J. Immunol. 1957, **78**, 387.
20. Halberg, F., Spink, W. W., and Bittner, J. J. Protection by aldosterone and 11,17-oxycorticoids against effects of brucella somatic antigen in adrenalectomized mice. Endocrinology 1956, **59**, 380.
21. Spink, W. W., and Hall, W. H. The influence of cortisone and adrenocorticotrophic hormone on brucellosis. II. Adrenocorticotrophic hormone (ACTH) in acute and chronic human brucellosis. J. clin. Invest. 1952, **31**, 958.
22. Magill, G. B., Killough, J. H., and Said, S. I. Cortisone and combined antibiotic therapy of acute brucellosis melitensis. Amer. J. Med. 1954, **16**, 810.
23. Spink, W. W. Adrenocorticotropic hormone and adrenal steroids in the management of infectious diseases. Ann. intern. Med. 1955, **43**, 685.

THE EFFECT OF ACUTE HYPOVOLEMIA ON THE RELEASE OF "ALDOSTERONE" AND ON THE RENAL EXCRETION OF SODIUM [1]

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Automatic control of the volume of the body fluids, involving adjustment of the renal excretion of electrolytes and water, has been the subject of much recent study. Accumulating evidence suggests that a variety of functions of the extracellular fluid volume are significant determinants of the renal excretion of sodium (2). Some investigators have emphasized the role of "effective" blood volume (or a related parameter) as such a function (3–6), but the precise stimuli involved and the mode of their transmission to the kidney remain incompletely understood.

The recent demonstration that aldosterone, a natural salt-retaining steroid of the adrenal cortex (7), is released in health and disease in quantities which are correlated with alterations in sodium excretion (8) and which are competent to influence the reabsorption of sodium by the renal tubules (8, 9) lends support to the long-held view that such an adrenal secretion is an important element in the normal government of body sodium (3, 10–12). Other investigations have shown that, in fact, the effect of changes in body fluid volume upon the renal excretion of sodium may be mediated in part by a graded secretion of aldosterone by the adrenal cortex (13–16).

The investigation reported here represents an attempt to define further certain aspects of the homeostatic reflexes involved in these processes in active humans, by an examination of the nature of some of the stimuli initiated by acute blood loss with its subsequent effect on the possible role of serum electrolytes and of aldosterone in the transmission of these stimuli and the mechanism of response of the end-organ effector, the kidney.

[1] A preliminary report of these studies has appeared, in part, elsewhere (1).

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METHODS

Experimental plan

Studies were performed upon two normally active, healthy humans (D. F., male, age 32; T. A., female, age 32), in whom unusual circulatory adjustments and losses of fluid via the skin were minimized by the proscription of strenuous exercise and the performance of studies during cool weather (October, 1955 to April, 1956). Observation on the female subject was made only during the first portion of the menstrual cycle.

A constant daily oral intake of food and fluid, supplemented by fixed amounts of sodium chloride, was begun prior to any observations and was maintained during the course of each study.

Each 24 hour study day commenced at 9 A.M. in the fasting state. Following three or more days of control observations, at 9 A.M. of each study day blood was removed by phlebotomy within an average of 20 minutes with the subject supine. In some instances, this was followed by a specific intravenous infusion (*vide infra*). Immediately thereafter, normal activity was resumed without significant symptoms and observations were continued for the ensuing four to five days. Following this, the experiment was ended and the blood previously removed was reinfused.

The studies were divided into three groups in accord with the basic procedure performed:

Group I—Simple phlebotomy. Four hundred fifty to 670 ml. of whole blood were removed by phlebotomy. Three studies were performed on each subject (D. F.—A, B, C; T. A.—a, b, c). In an attempt to eliminate any differences between these studies and those in Group II, arising solely from the effect of the sodium (14 to 20 mEq.) contained in the 25 per cent albumin infusions used in Group II, a similar amount of sodium (in normal saline) was infused following phlebotomy in a few of the Group I studies (I-A, I-B, I-b).

Group II—Phlebotomy plus 25 per cent albumin. Four hundred seventy-five to 660 ml. of whole blood were removed by phlebotomy. The intravenous infusion of a 25 per cent solution of salt-poor human albumin [3] was begun immediately thereafter and was completed within

[3] The albumin solutions employed in these studies were generously provided by the New York Regional Blood Program of the American Red Cross.

20 minutes following phlebotomy. The amount of albumin employed was expected to produce an expansion of the plasma volume equal to the volume of whole blood withdrawn by phlebotomy, based upon the assumption that on the average one gram of injected albumin will draw 18 ml. of extravascular fluid into the circulation (17). Two studies were performed on each subject (D. F.—A, B; T. A.—a, b).

Group III—Phlebotomy plus 25 per cent albumin plus saline. Following a 590 ml. phlebotomy, a 25 per cent solution of albumin and a normal solution of saline were infused intravenously over a 50 minute period. The quantity of albumin infused was determined as in Group II. The volume of saline solution plus the volume of albumin solution equalled the volume of blood withdrawn by phlebotomy. One study was performed on Subject D. F.

Measurements

Fasting body weight was determined periodically by weighing the subject in the same clothes after the 9 A.M. voiding. At appropriate intervals, venous blood was withdrawn without stasis and measurements were made of hemoglobin concentration (18) and hematocrit (19), and of the concentration of sodium, potassium and creatinine chromogen (20) in the serum. Fasting blood for electrolyte determinations was obtained under oil. Urine was refrigerated during collection, and, after measurement of the 24 hour volume, aliquots were set aside for the determination of creatinine chromogen (20), sodium and potassium. The remainder was preserved at — 20° C., for later measurement of 17-hydroxycorticosteroids (21), 17-ketosteroids (22), and for bioassay of salt-retaining activity. Saliva collections were obtained before 9 A.M. in the fasting state (23) for the measurement of sodium and potassium concentration. For Subject D. F., some direct assessment of the average daily fecal electrolyte excretion was obtained by analysis of incinerated aliquots of a homogenized five and six day pooled specimen, collected during two separate studies. The content of sodium, potassium and water in each constant diet was determined by analysis of aliquots of a homogenized duplicate 24 hour diet. All sodium and potassium determinations were made on a flame photometer using an internal Lithium standard.

Bioassay of salt-retaining activity of urine, based upon the effect of injected extracts upon the urinary potassium: sodium ratio of adrenalectomized rats, was performed by modification of the method of Johnson (24).[4] Extracts were prepared by the method of Axelrad, Cates, Johnson, and Luetscher (25) (using hydrolysis A and B combined), but without chromatographic partition. The results are referred to as "aldosterone" and expressed as *gamma DCA equivalents* (GDE), since true aldosterone was not actually measured. However, the results of assay of unchromatographed extracts do not usually diverge markedly from those of assay of "fraction E" (25). Since the 17-hydroxycorticosteroids were never abnormally elevated in these urines, the possibility of interfering effects was probably lessened (24). A control and postphlebotomy urine from the same study were always extracted and assayed simultaneously. The postphlebotomy urinary "aldosterone" excretion was considered to be significantly elevated if it exceeded the normal range of control values for all studies in that subject (*i.e.*, mean of all controls for that subject plus or minus two times S. D.) and if the "aldosterone" effect of the postphlebotomy urine extract and of the paired control urine extract differed significantly in the bioassay (*i.e.*, if $p < 0.05$ for the difference of means of the potassium: sodium ratios of the rat urines in the bioassay of each extract).

Calculations

The average glomerular filtration rate over a 24 hour period was estimated by the endogenous creatinine chromogen clearance (26), calculated from the total chromogen excretion and the mean of creatinine chromogen concentrations of three to four serum specimens obtained during this period.

Each 24 hour urine volume was used as an estimate of daily water excretion.

A crude assessment of changes in plasma and blood volume associated with phlebotomy was obtained as follows:

$$BV_0 = 73 \times BW \text{ (27)}$$

$$BV_1 = BV_0 - V$$

$$BV_n = BV_1 \times \frac{Hb_1}{Hb_n}$$

$$\Delta BV_n = BV_n - BV_0$$

$$PV_n = BV_n \times (1 - Ht_n)$$

$$\Delta PV_n = PV_n - PV_0$$

where V is the volume of blood removed at phlebotomy (ml.), BV is the total blood volume (ml.), PV is the total plasma volume (ml.), Hb is the hemoglobin concentration (grams per cent), Ht is the hematocrit per 100, BW is the body weight (kilograms), Δ is the change from prephlebotomy value, and where subscript 0 is the value immediately before phlebotomy, subscript 1 is the value immediately after, and subscript n is the value at specific time after completion of phlebotomy.

Because complete balances were not determined, variations in daily renal excretion are presented as deviations (plus or minus) from the mean of the daily urinary excretion during the control period in each study. Since a 24 hour excretion rate, which was less than the mean control rate, represented the amount retained over and above the mean control, it is referred to as "renal conservation." When the excretion rate exceeded the mean control value, it is referred to as "renal loss." The effect of the phlebotomy procedure upon the excretion

[4] The desoxycorticosterone acetate (DCA) employed as a standard in this assay was kindly provided by the Schering Corporation, Charles Pfizer and Company, and the Ciba Pharmaceutical Company.

TABLE I

The intake and renal excretion of sodium, potassium and water in two active subjects during control periods and following phlebotomy procedures

Subject—Study		Phlebotomy—Group I																					
		T. A. I-a			T. A. I-b			T. A. I-c			D. F. I-A			D. F. I-B			D. F. I-C		
Daily intake*†‡		Na 193	K 75	H_2O C§	Na 201	K 78	H_2O 1,615	Na 185	K 71	H_2O C§	Na 197	K 101	H_2O 1,930	Na 197	K 101	H_2O 1,930	Na 197	K 101	H_2O 1,930
Renal excretion*— control days	−5							183	69	1,300	162	92	930	177	95	1,195	253	74	1,510
	−4				190	68	905	155	67	780	216	96	1,135	215	79	1,125	168	80	960
	−3	161	62	1,460	178	53	640	188	64	1,250	171	91	1,110	211	91	1,145	165	80	1,050
	−2	192	82	2,000	187	59	980	173	61	1,340	205	88	1,295	197	94	1,220	198	81	1,410
	−1	174	75	1,520	209	65	1,105	176	71	1,100	224	83	1,422	213	97	1,101	192	101	960
	Mean	176	73	1,660	191	61	908	175	66	1,154	196	90	1,178	203	91	1,157	195	83	1,178
	S.D.	±16	±10	±296	±13	±7	±197	±13	±4	±228	±28	±5	±188	±16	±7	±49	±35	±10	±262
Procedure		Phlebotomy 450 ml.			Phlebotomy 450 ml.			Phlebotomy 670 ml.			Phlebotomy 610 ml.			Phlebotomy 600 ml.			Phlebotomy 600 ml.		
Renal excretion*— days following procedure	+1	112	76	1,180	145	63	830	116	57	890	105	95	854	122	93	818	130	97	830
	+2	160	87	1,680	202	76	760	124	57	955	111	88	873	144	97	950	164	87	840
	+3	174	68	1,680	192	67	695	160	70	890	190	103	1,013	171	75	1,030	189	90	1,100
	+4	198	56	1,350	215	64	1,185	190	78	1,460	197	84	980	242	78	1,020	200	103	1,080
	+5				204	73	1,185	182	66	1,100	217	84	1,020	211	95	990	187	95	1,095

Subject—Study		Phlebotomy + 25% Albumin—Group II									Phlebotomy + 25% Albumin + Saline—Group III					
		T. A. II-a			T. A. II-b			D. F. II-A			D. F. II-B			D. F. III		
Daily intake*†‡		Na 201	K 78	H_2O 1,615	Na 185	K 71	H_2O C	Na 197	K 101	H_2O 1,930	Na 197	K 101	H_2O 1,930	Na 197	K 101	H_2O 1,930
Renal excretion*— control days	−5				160	55	880				208	92	1,110	218	85	1,395
	−4	152	70	800	160	59	1,082	202	92	1,000	191	100	1,015	229	91	1,225
	−3	187	65	805	219	66	1,450	203	102	1,075	179	100	980	219	86	1,560
	−2	155	65	800	194	71	1,580	189	83	1,065	226	88	1,425	181	106	1,185
	−1	173	53	740	168	67	1,240	171	84	1,017	181	94	985	185	93	970
	Mean	167	63	786	180	64	1,246	191	90	1,039	197	95	1,103	206	92	1,267
	S.D.	±16	±7	±31	±26	±7	±280	±15	±9	±36	±20	±5	±187	±22	±8	±224
Procedure		Phlebotomy 475 ml. 25% albumin 100 ml. I.V.			Phlebotomy 660 ml. 25% albumin 147 ml. I.V.			Phlebotomy 620 ml. 25% albumin 136 ml. I.V.			Phlebotomy 615 ml. 25% albumin 137 ml. I.V.			Phlebotomy 590 ml. 25% albumin 135 ml. + normal saline 465 ml. I.V.		
Renal excretion*— days following procedure	+1	124	56	633	124	58	680	183	87	922	130	100	845	223	111	1,275
	+2	160	72	820	138	64	1,130	166	77	1,130	161	98	885	211	103	1,080
	+3	186	66	895	216	66	1,320	94	104	795	199	80	955	165	105	920
	+4	170	73	845	163	61	980	159	92	883	174	80	1,280	179	77	1,000
	+5	212	76	975	144‖	48‖	950‖	172	84	940	195	84	1,155	219	84	1,450

* Milliequivalents or cubic centimeters per day.

† Constant intake was begun one to nine days before control observations were started and was continued throughout each study, except that sodium and water intake was augmented during day +1 in studies I-A, I-B, I-b, where a small amount of normal saline was infused and studies II-A, II-B, II-a, II-b and III, where 25% albumin (+saline in III) was infused immediately after phlebotomy. The additional sodium contained in these infusions was 19, 18, 14, 19, 19, 14, 20 and 90 milliequivalents, respectively.

‡ Water intake represents total water content of ingested food and fluids.

§ Daily water intake was constant, as in all studies, but performed water was not determined.

‖ Severe diarrhea.

of sodium, potassium and water is estimated by the cumulative "conservation" or "loss" of these substances during the consecutive days, immediately following phlebotomy, in which the excretion rate deviated from the mean control value in a consistently positive or negative direction.

A rough assessment of balance during the control periods is made on the basis of the assumption that in Subject D. F. the average daily fecal sodium and potassium equalled the mean of that in the two pooled specimens (sodium equals 0.4 mEq. per day, and potassium equals 6 mEq. per day) and that in Subject T. A. the daily fecal sodium and potassium corresponded to the range found in human subjects on a variety of diets (sodium equals 4 ± 4 mEq. per day, and potassium equals 10 ± 5 mEq. per day) (28). All stools were formed except on the final day of one study (II-b).

RESULTS

Group I—Simple phlebotomy (Table I and Figure 1)

Blood volume. Hemodilution occurred to only a limited extent by eight hours, but essentially restored prephlebotomy blood volume by 24 to 48 hours after the phlebotomy (mean deviation from prephlebotomy blood volume was −120 ml. at 24 hours and −40 ml. at 48 hours). That the ex-

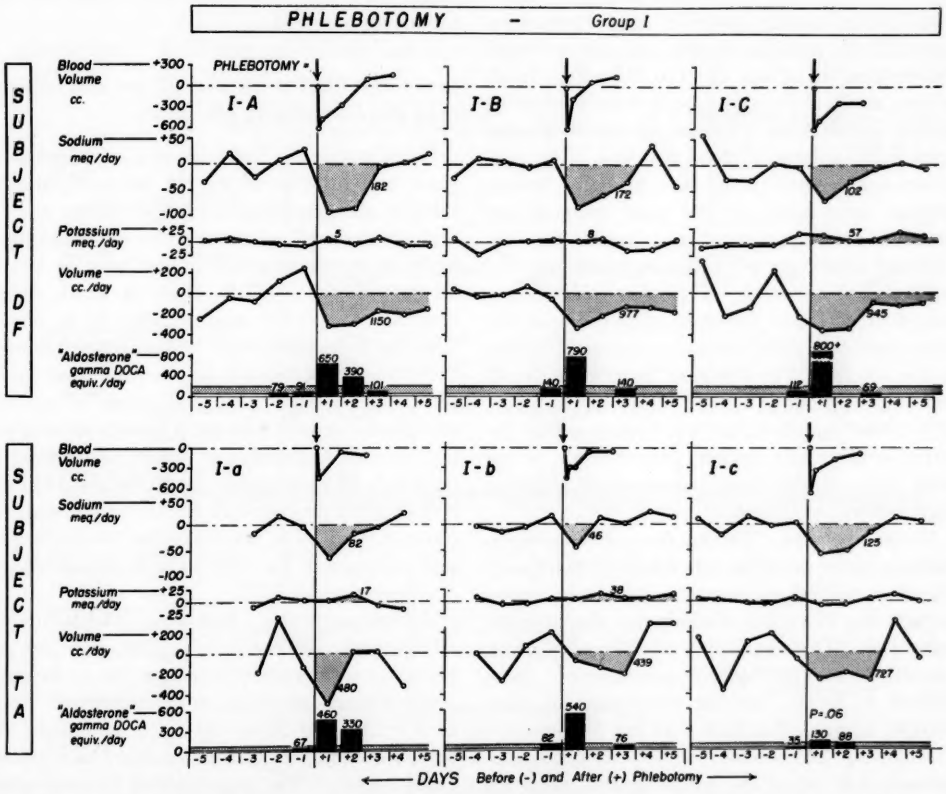

FIG. 1. THE EFFECT OF SIMPLE PHLEBOTOMY ON BLOOD VOLUME, URINE VOLUME AND URINARY SODIUM, POTASSIUM, AND "ALDOSTERONE"

Blood volume is depicted as the deviation from assumed prephlebotomy value. Urinary volume, sodium and potassium are depicted as deviations from the mean prephlebotomy control value for each study and shaded areas with adjacent figures represent cumulative "renal conservation" of sodium and water and cumulative "renal loss" of potassium following phlebotomy. Urinary "aldosterone" rise after phlebotomy which did not differ significantly from prephlebotomy value is indicated by $p < 0.05$. Horizontal shaded area indicates mean of all control "aldosterone" values plus or minus two times S. D. for each subject.

pansion of plasma volume in response to blood loss can account for the replacement of red blood cell volume as well as plasma volume has been noted previously (29) and suggests that regulation of plasma volume is subservient to the restoration of *total* blood volume, rather than to the maintenance of plasma volume alone. The necessary expansion of plasma volume is attained under these circumstances by the acquisition of fluid from extravascular sources, induced by the oncotic effect of plasma protein derived from endogenous stores (29).

Sodium excretion. Following phlebotomy, renal sodium excretion was reduced below the mean control value in every study for one to three days consecutively, resulting in the cumulative "renal conservation" of sodium of 46 to 182 mEq. In all but one study (I-b), this exceeded the amount of sodium contained in a volume of normal plasma equal to the volume of blood removed. The conservation of sodium noted here in active human subjects corresponds to the same phenomenon observed previously in normal supine humans (30) and in patients (4) following blood loss.

Potassium excretion. Following phlebotomy, potassium excretion increased slightly above the mean control value for one to five consecutive days in five studies. The resultant cumulative "renal loss" of potassium of 5 to 57 mEq. was considerably less in magnitude, in most instances, than the paired simultaneous sodium conservation. In one study (I-c), slight "renal conservation" of potassium followed phlebotomy.

Water excretion. During the control periods, the daily water excretion was relatively more variable than electrolyte excretion in most instances, perhaps due to greater viscissitudes of extrarenal water loss. The significance of changes in water excretion after phlebotomy are therefore more difficult to assess, but the consistency of these changes suggests that they were not fortuitous.

Following phlebotomy, water excretion consistently fell below the mean control excretion (although not below the mean minus two times S. D.) for one to five consecutive days, resulting in cumulative "renal conservation" of water of 439 to 1,150 ml. during this period. This volume was approximately equal to or greater than the volume of the phlebotomy in each instance.

"Aldosterone." The normal range of the control urinary "aldosterone" was 50 ± 40 GDE per 24 hours for all studies in Subject T. A., and 110 ± 80 GDE per 24 hours for all studies in Subject D. F. During the first 24 hours after phlebotomy, urinary "aldosterone" rose significantly above the prephlebotomy control value in five studies to levels of 460 to 800 plus GDE per 24 hours. Where it was measured, the urinary "aldosterone" was elevated on the second day (I-A, I-a) and returned to the normal control range on the third day (I-A, I-B, I-C, I-b). In the one study (I-c) in which the postphlebotomy "aldosterone" rise was not considered to be significant, the largest phlebotomy was performed and a marked antinatriuresis occurred.

Group II—Phlebotomy plus 25 per cent albumin (Table I and Figure 2)

Blood volume. Following the withdrawal of blood and infusion of the 25 per cent albumin solution, the prephlebotomy blood volume was essentially restored or exceeded in less than three hours in every study and in one hour or less in the two instances (II-B, II-b) in which earlier determinations were made. This is in accord with the observation that hemodilution following such albumin infusions is largely completed within 15 minutes (17). The replacement of red cell and plasma volume here must have been achieved by the same acquisition of extravascular fluid as occurred in spontaneous hemodilution (I), except that the process was greatly accelerated, because the immediate administration of plasma protein supplanted the slower mobilization of endogenous protein (17).

Sodium excretion. Following phlebotomy and albumin infusion, the daily sodium excretion fell below the mean control value for two to five consecutive days, resulting in a cumulative "renal conservation" of 50 to 181 mEq. of sodium, despite the rapid restoration of initial blood volume (*vide supra*). The magnitude of "conservation" approximated that following simple phlebotomy (I), but the pattern of antinatriuresis was less consistent. In three studies (II-B, II-a, II-b) the lowest sodium excretion occurred during the first day, although the sodium intake on that day exceeded the constant intake of other days, due to the sodium content (14 to 20 mEq.) of the infused

25 per cent albumin solution. However, in one study (II-A) the maximum antinatriuresis occurred after the first 48 hours, despite the restoration of blood volume by two and one-half hours after venisection.

Potassium excretion. Following phlebotomy and albumin infusion, there was no consistent or significant change in potassium excretion.

Water excretion. During the control periods, water excretion was again somewhat variable. Following phlebotomy and albumin infusion, water excretion was reduced below the mean control value in every instance for one to three consecutive days, resulting in a cumulative "renal conservation" of water of 117 to 682 ml.

"Aldosterone." Following phlebotomy and albumin infusion, the urinary "aldosterone" rose above the control range for each subject in association with the maximum antinatriuresis, although this rise was not considered to be significant in a study (II-b) in which the largest phlebotomy was performed and in which a marked antinatriuresis occurred. In each subject, the significant elevations were more modest than after simple phlebotomy (I), although this difference may not be meaningful.

Group III—Phlebotomy plus 25 per cent albumin plus saline (Table I and Figure 2)

Following phlebotomy and infusion of the albumin and saline solutions, blood volume was found to surpass the prephlebotomy value at the

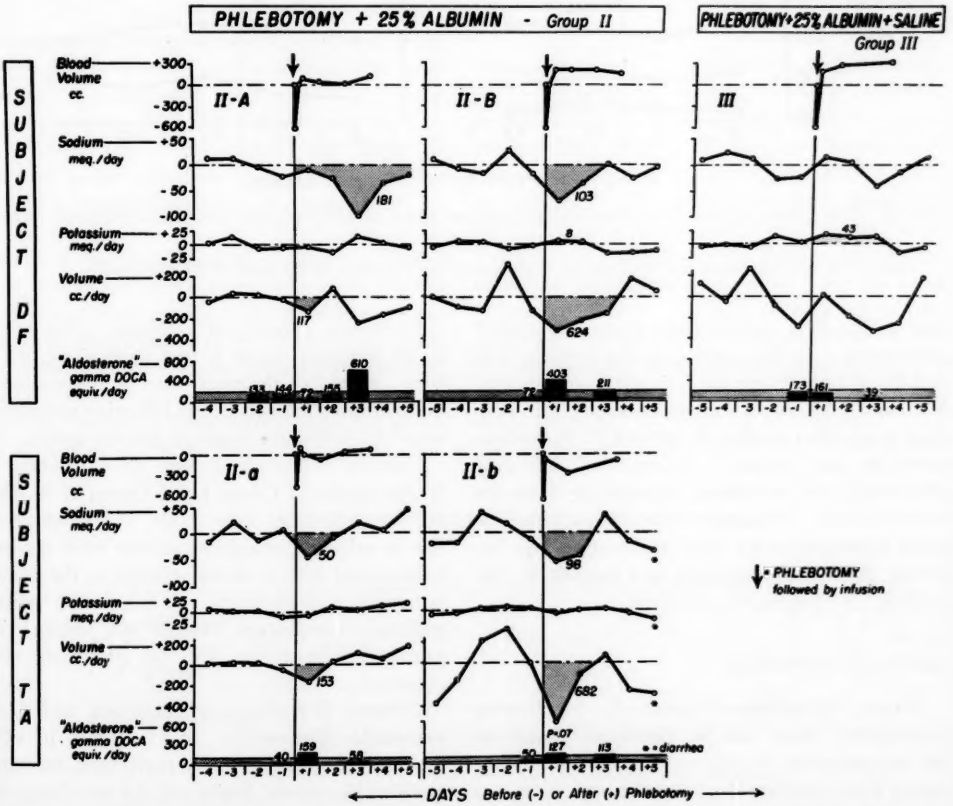

FIG. 2. THE EFFECT OF PHLEBOTOMY PLUS 25 PER CENT ALBUMIN INFUSION (PLUS OR MINUS SALINE INFUSION) ON BLOOD VOLUME, URINE VOLUME, AND URINARY SODIUM, POTASSIUM, AND "ALDOSTERONE"

Graphic representation as in Figure 1.

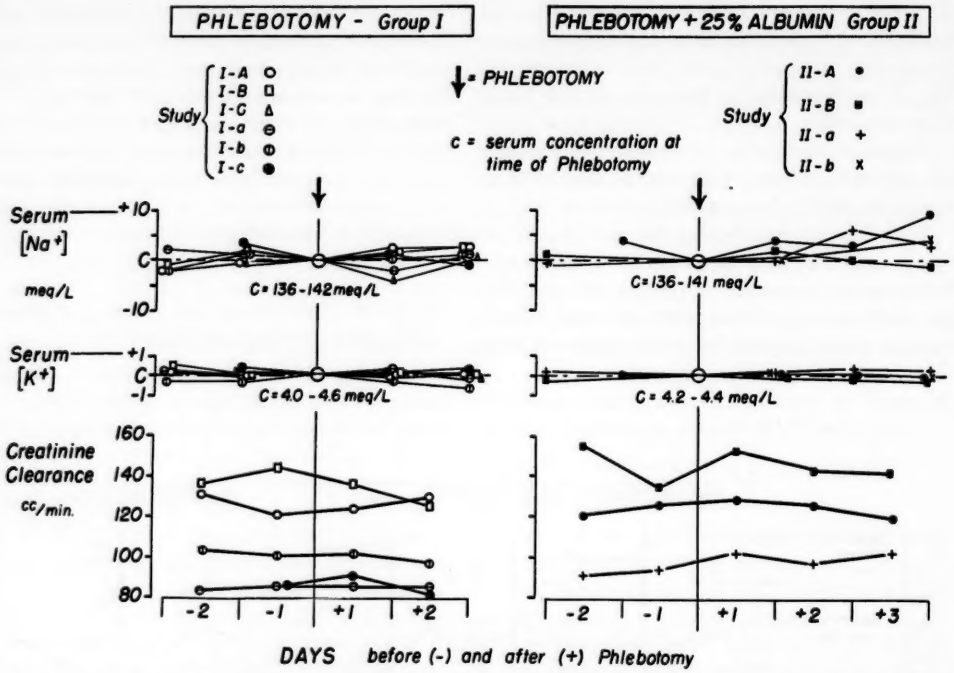

FIG. 3. THE EFFECT OF THE PHLEBOTOMY PROCEDURE ON THE CONCENTRATION OF SERUM ELECTROLYTES AND ON THE ENDOGENOUS CREATININE CLEARANCE

Electrolyte concentrations are depicted as deviations from the value at the time of the phlebotomy.

time of the first measurement three hours after phlebotomy. In this study both the isotonic fluid and the plasma protein for hemodilution were rapidly supplied from an exogenous source. In contrast to all other studies in Subject D. F., sodium excretion and urinary "aldosterone" following phlebotomy did not change significantly from the control values. Potassium excretion exceeded the mean control value for three consecutive days following phlebotomy, resulting in a cumulative "renal loss" of 43 mEq. of potassium.

Additional observations

Serum electrolytes (*Figure 3*). Following phlebotomy, there was no significant change in the concentration of sodium or potassium in the serum from the control values, although there was a tendency for the sodium concentration to rise slightly following the phlebotomy and albumin infusion (II).

Creatinine chromogen clearance (*Figure 3*). In all instances where it was determined, the 24 hour creatinine clearance during the period of the maximum antinatriuresis following phlebotomy never fell below the range of control values.

Salivary potassium: sodium ratio (*Figure 4*). In the studies of Group I and Group II in which salivary collections were made, a postphlebotomy rise in salivary potassium: sodium ratio occurred concomitant with a similar change in the urinary potassium: sodium ratio. A fall in the salivary sodium concentration (values not shown) was primarily responsible for the increased ratios observed.

Urinary 17-hydroxycorticosteroids and 17-ketosteroids (*Figure 5*). In all studies in which steroid determinations were performed, the values were within normal limits and did not change significantly in association with the "aldosterone" increases.

Electrolyte balance during control periods.

Based upon the assumptions discussed under the Methods section, the mean daily balance of sodium and potassium during the control period of all studies varied from slightly negative to moderately positive.

Body weight. During the entire period in which these studies were performed, Subject D. F. weighed 73.6 to 76.4 kilograms and Subject T. A. weighed 52.0 to 54.6 kilograms. The greatest change in weight during any one study was 0.8 kilogram. Therefore, excluding gross variations in water balance, it may be assumed that major changes in body composition did not occur during these experiments.

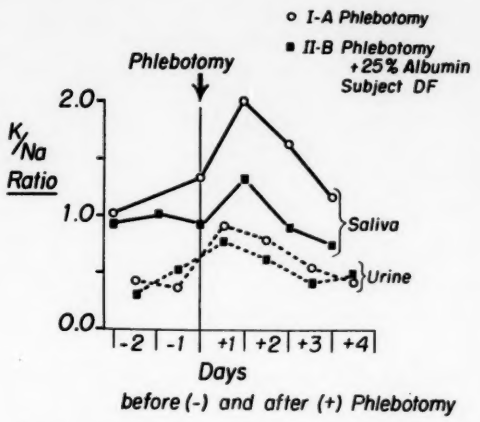

FIG. 4. THE RELATIONSHIP OF THE POTASSIUM: SODIUM RATIO IN SALIVA TO THAT IN URINE BEFORE AND AFTER PHLEBOTOMY

DISCUSSION

An acute reduction in the volume of the blood clearly initiated processes which culminated in a moderate conservation of sodium and water by the kidney, a less intense and less consistent augmentation of potassium excretion and a fairly consistent elevation of urinary "aldosterone."

Our data support the concept that these alterations in the excretion of electrolytes can be attributed predominantly to modification of renal tubular function (4, 30) rather than to alteration of the loads of these electrolytes filtered at the glomerulus, since there were no significant alterations of the concentrations of sodium and potassium in the serum or of the endogenous creatinine chromogen clearances following phlebotomy. The view that the fairly consistent relationship between the increased "aldosterone" and the modified electrolyte excretion was causal, rather than merely coincident, is supported by a consideration of the quantitative aspects of these responses. It seems reasonable to assume that here, as in other studies (8, 14, 15), urinary "aldosterone" reflected the level of circulating aldosterone. The elevated quantities of "aldosterone" found following the removal of blood in most of our Group I studies were roughly of the same order of magnitude as the amounts recovered from the urine of human subjects, with and without adrenal insufficiency, following the administration of 600 to 1,250 gamma of aldosterone per day (8, 31) [assuming that aldosterone is between 30 and 40 times as potent as DCA in the bioassay (8)]. The exhibition of such quantities of aldosterone

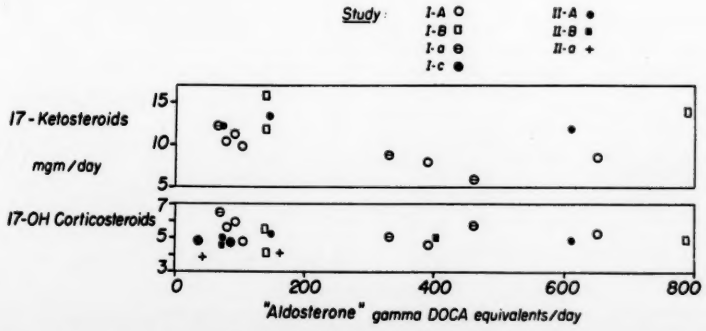

FIG. 5. THE RELATIONSHIP OF URINARY 17-KETOSTEROIDS AND 17-HYDROXYCORTICOSTEROIDS TO URINARY "ALDOSTERONE"

to humans, without adrenal insufficiency, results in a moderate decrement in sodium excretion and a generally lesser or absent increment in potassium excretion by the kidney (31–33), which approximates the dimensions and configuration of the renal response in our subjects following simple phlebotomy (1). Additional support for the assumption that the elevation of urinary "Aldosterone" reflected a significant rise in circulating aldosterone is provided by the *pari passu* rise in the potassium : sodium ratio in the salivary secretion, a function which has been observed to respond in such a fashion to exogenously administered aldosterone (7). Although significant elevations of urinary "aldosterone" in studies of Group II tended to be of a smaller magnitude than in those of Group I, it seems reasonable to assume that they also indicated an increased circulating level.

The marked renal conservation of sodium associated with the largest phlebotomies in Subject T. A. (I-c, II-b) in the absence of a large or significant elevation of the urinary "aldosterone" is not explained by the mechanisms considered above. In study I-c the data do not suggest a fall in the glomerular filtration rate which could produce a discordance between urinary and circulating steroids (34). In all of these studies, reductions in the glomerular filtration rate may have occurred which were too small to detect by the method employed and which could have been responsible for significant sodium retention by the kidney (35). In addition, reduction of blood volume and its sequelae may, like other stimuli, accelerate the tubular reabsorption of sodium independently of variations in salt-retaining steroid (36).

The water conservation observed following simple phlebotomy (1) also appears to have stemmed primarily from an enhanced tubular reabsorption of water, as indicated by the stable creatinine clearances. Our experiments do not provide data to suggest whether this effect was mediated by antidiuretic hormone (ADH) or by mechanisms which operate independently of this hormone (37). A competent stimulus for the release of ADH was present as some function of decreased fluid volume (38), and may have been present as a rising tonicity (39) resulting from the primary conservation of sodium, although the latter may have been obscured by secondary water retention.

The rise in "aldosterone" noted in our studies, without a concurrent rise in other urinary corticosteroids, resembles the dissociation seen in many situations where an intense stimulus for sodium conservation exists (40). The observation that blood loss in the dog produces an increase in the aldosterone in adrenal venous blood, which is greater proportionately than the increase in hydrocortisone (41), suggests that the increased urinary "aldosterone" noted in our studies was due to augmented adrenal secretion of aldosterone, although our data do not exclude the possibility that it derived from a diminished rate of peripheral destruction. If such an increased secretion did in fact occur, it is unlikely that adrenocorticotropic hormone (ACTH) was the responsible stimulus, since this hormone produces a marked elevation of 17-hydroxycorticosteroid release but exerts little effect upon the release of aldosterone (7, 40). It also is unlikely that an elevation of the serum potassium concentration or potassium : sodium ratio provided a stimulus for aldosterone release (42), since these parameters were not significantly altered following phlebotomy, although it is possible that small, transient changes were obliterated by secondary renal responses.

The nature of the primary stimuli which lead to an augmented aldosterone release and to an antinatriuresis is of considerable interest. The absence of sodium conservation when the removed blood was replaced with 25 per cent albumin and saline (Group III) suggests that neither the stress of the phlebotomy nor the loss of red blood cells *per se* provided an adequate stimulus. The response to simple phlebotomy (Group I), however, is compatible with the concept that a decrease in blood volume, pressure or flow in some area of the intravascular space can produce an effective stimulus (3–6).

On the other hand, the retention of sodium and the "aldosterone" rise which occurred despite the rapid restoration of initial blood volume, when phlebotomy was followed by the infusion of a 25 per cent albumin solution (Group II), suggests that a sustained reduction of blood volume is not the exclusive stimulus. It seems unlikely that the evanescent reduction of blood volume which occurred here (II) was itself an effective

stimulus, because it was of such short duration, because the major effector response did not occur until two days after the phlebotomy in one study (II-A), and since no such response appeared when blood volume was restored by the 25 per cent albumin-saline plasma substitute (III) over a comparable period. Since the initial reexpansion of blood volume must have been attained by the rapid uptake of an appropriate volume of essentially isotonic fluid from an extravascular locus, probably the interstitial space (43), it seems reasonable to conjecture that reduction of that space (or some function thereof) may provide a stimulus which is monitored by some suitable local receptors.

In view of the fact that hyperoncotic albumin infusions are in themselves antinatriuretic in normal and adrenal insufficient subjects (44), it is not certain that the mechanisms responsible for the sodium conservation observed when 25 per cent albumin infusions restored blood volume (II) were the same as those responsible for the sodium conservation noted after blood loss and spontaneous hemodilution (I). However, since an initial rise in oncotic pressure following 25 per cent albumin infusion must have been rapidly extinguished by the movement of fluid into the vascular compartment, it would seem less likely that the stimulus of a rising oncotic pressure was involved here, than the stimulus of a diminishing interstitial fluid volume (44).

The concept of a stimulus arising from a decrease in the interstitial volume is not incompatible with the fact that an aldosterone rise has been observed following blood removal, even when the plasma sodium and water removed were replaced by a normal saline infusion (9), since restoration of the *total* blood volume, not just of the plasma volume alone, is the dominant physiological desideratum in the hemodilution process (29) and would have required the accretion of additional isotonic fluid from extravascular sources to replace the red cell volume removed. Inferences drawn from a variety of other studies (2, 44, 45) have suggested that a decrease in interstitial fluid volume in some area may be one of several initial stimuli for the renal conservation of sodium, regardless of how it is transmitted to the kidney.

In these studies, the effect of sodium lost in phlebotomy was not controlled by observing the effect of sodium replacement alone. Therefore, it cannot be concluded from our data that the sodium conservation after blood loss occurs by mechanisms which differ from those operative following the deprivation of dietary sodium (8). In both of these situations, the initial stimulus may be the absolute loss of body sodium or the simultaneous decrement of portions of the extracellular fluid volume.

SUMMARY

The effect of acute hypovolemia, induced by phlebotomy, was observed in two normal active human subjects maintained on constant diets.

When hemodilution occurred spontaneously, there was a consistent reduction of urinary sodium, associated with a fairly consistent elevation of urinary "aldosterone." Similar changes in sodium and "aldosterone" excretion prevailed when the blood volume was rapidly restored by the immediate infusion of 25 per cent salt-poor human albumin.

Following phlebotomy, there was no significant alteration in the endogenous creatinine chromogen clearance, the urinary 17-hydroxycorticosteroids and 17-ketosteroids, or in the concentration of sodium and potassium in the serum.

From these observations, it appears that blood loss and the associated hemodilution process initiate changes which may be monitored in the extravascular space as well as in the intravascular space, and which culminate in the renal conservation of sodium, probably due, in part, to the accelerated tubular reabsorption of this ion.

The renal response seems to be mediated, in part, by an increase in the effective level of circulating aldosterone, which does not depend upon ACTH or changes in the potassium : sodium ratio in the serum.

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REFERENCES

1. Fine, D., Meiselas, L. E., and Auerbach, T. The relationship of acute reduction in blood volume to the release of "aldosterone." Clin. Res. Proc. 1956, **4**, 126.
2. Grossman, J. Volume factors in body fluid regulation. Arch. intern. Med. 1957, **99**, 93.
3. Peters, J. P. The role of sodium in the production of edema. New Engl. J. Med. 1948, **239**, 353.
4. Borst, J. G. G. The maintenance of an adequate cardiac output by the regulation of the urinary excretion of water and sodium chloride; an essential factor in the genesis of oedema. Acta med. scand. 1948, Suppl. **207**.
5. Wilkins, R. W., Tinsley, C. M., Culbertson, J. W., Burrows, B. A., Judson, W. E., and Burnett, C. H. The effects of venous congestion of the limbs upon renal clearances and the excretion of water and salt. I. Studies in normal subjects and in hypertensive patients before and after splanchnicectomy. J. clin. Invest. 1953, **32**, 1101.
6. Epstein, F. H. Renal excretion of sodium and the concept of a volume receptor *in* Essays in Metabolism: The John Punnett Peters Number of the Yale Journal of Biology and Medicine, L. G. Welt, Ed. Boston, Little, Brown & Co., 1957, pp. 282–298.
7. Simpson, S. A., and Tait, J. F. Recent progress in methods of isolation, chemistry, and physiology of aldosterone *in* Recent Progress in Hormone Research, G. Pincus, Ed. New York, Academic Press Inc., 1955, vol. II, pp. 183–210.
8. Luetscher, J. A., Jr., and Curtis, R. H. Observations on aldosterone in human urine. Fed. Proc. 1955, **14**, 746.
9. Bartter, F. C. The role of aldosterone in normal homeostasis and in certain disease states. Metabolism 1956, **5**, 369.
10. Loeb, R. F., Atchley, D. W., Benedict, E. M., and Leland, J. Electrolyte balance studies in adrenalectomized dogs with particular reference to the excretion of sodium. J. exp. Med. 1933, **57**, 775.
11. Conn, J. W., Johnston, M. W., and Louis, L. H. Acclimatization to humid heat: A function of adrenal cortical activity (abstract). J. clin. Invest. 1946, **25**, 912.
12. Leaf, A., and Couter, W. T. Evidence that renal sodium excretion by normal human subjects is regulated by adrenal cortical activity. J. clin. Invest. 1949, **28**, 1067.
13. Beck, J. C., Dyrenfurth, I., Giroud, C., and Venning, E. H. Observations on the regulatory mechanisms of aldosterone secretion in man. Arch. intern. Med. 1955, **96**, 463.
14. Duncan, L. E., Jr., Liddle, G. W., and Bartter, F. C. The effect of changes in body sodium on extracellular fluid volume and aldosterone and sodium excretion by normal and edematous men. J. clin. Invest. 1956, **35**, 1299.
15. Bartter, F. C., Liddle, G. W., Duncan, L. E., Jr., Barber, J. K., and Delea, C. The regulation of aldosterone secretion in man: The role of fluid volume. J. clin. Invest. 1956, **35**, 1306.
16. Muller, A. F., Riondel, A. M., and Mach, R. S. Control of aldosterone excretion by changes in volume of body-fluid. Lancet 1956, **1**, 831.
17. Heyl, J. T., Gibson, J. G., 2nd, and Janeway, C. A. Studies on the plasma proteins. V. The effect of concentrated solutions of human and bovine serum albumin on blood volume after acute blood loss in man. J. clin. Invest. 1943, **22**, 763.
18. Crosby, W. H., Munn, J. I., and Furth, F. W. Standardizing a method for clinical hemoglobinometry. U. S. armed Forces med. J. 1954, **5**, 693.
19. Wintrobe, M. M. Macroscopic examination of the blood: Discussion of its value and description of the use of a single instrument for the determination of sedimentation rate, volume of packed red cells, leukocytes and platelets, and of icteric index. Amer. J. med. Sci. 1933, **185**, 58.
20. Bonsnes, R. W., and Taussky, H. H. On the colorimetric determination of creatinine by the Jaffe reaction. J. biol. Chem. 1945, **158**, 581.
21. Silber, R. H., and Porter, C. C. Determination of 17, 21-dihydroxy-20-ketosteroids in urine and plasma. J. biol. Chem. 1954, **210**, 923.
22. Drekter, I. J., Pearson, S., Bartczak, E., and McGavack, T. H. Rapid method for determination of total urinary 17-ketosteroids. J. clin. Endocr. 1947, **7**, 795.
23. Frawley, T. F., and Thorn, G. W. Relation of salivary sodium-potassium ratio to adrenal cortical activity *in* Proceedings of the Second Clinical ACTH Conference, vol. I., Research, J. R. Mote, Ed. Philadelphia, Blakiston, Co. 1951, pp. 115–122.
24. Johnson, B. B. Bioassay of adrenal cortical steroids on the basis of electrolyte excretion by rats: Effects of 11-desoxy and 11-oxy-steroids. Endocrinology 1954, **54**, 196.
25. Axelrad, B. J., Cates, J. E., Johnson, B. B., and Luetscher, J. A., Jr. Aldosterone in urine of normal man and of patients with oedema: Its increased recovery after hydrolysis with acid and with beta-glucuronidase. Brit. med. J. 1955, **1**, 196.
26. Sirota, J. H., Baldwin, D. S., and Villarreal, H. Diurnal variations of renal function in man. J. clin. Invest. 1950, **29**, 187.
27. Berson, S. A. Blood volume in health and disease. Bull. N. Y. Acad. Med. 1954, **30**, 750.
28. Danowski, T. S., and Greenman, L. Changes in fecal and serum constituents during ingestion of cation and anion exchangers. Ann. N. Y. Acad. Sci. 1953, **57**, 273.
29. Ebert, R. V., Stead, E. A., Jr., and Gibson, J. G., II. Response of normal subjects to acute blood loss. Arch. intern. Med. 1941, **68**, 578.

30. Lombardo, T. A., Eisenberg, S., Oliver, B. B., Viar, W. N., Eddleman, F. E., Jr., and Harrison, T. R. Effects of bleeding on electrolyte excretion and on glomerular filtration. Circulation 1951, **3**, 260.
31. Ward, L. E., Polley, H. F., Slocumb, C. H., Hench, P. S., Mason, H. L., Mattox, V. R., and Power, M. H. The effects of aldosterone (electrocortin) and of 9-alpha-fluorohydrocortisone acetate on rheumatoid arthritis: Preliminary report. Proc. Mayo Clin. 1954, **29**, 649.
32. Mach, R. S., and Fabre, J. Clinical and metabolic effects of aldosterone *in* Ciba Foundation Colloquia on Endocrinology. The Human Adrenal Cortex, G. Wolstenholme and M. Cameron, Eds. Boston, Little, Brown & Co., 1955, vol. VIII, pp. 361–372.
33. Thorn, G. W., Sheppard, R. H., Morse, W. I., Reddy, W. J., Beigelman, P. M., and Renold, A. E. Comparative action of aldosterone and 9-alpha-fluorohydrocortisone in man. Ann. N. Y. Acad. Sci. 1955, **61**, 609.
34. Marks, L. J., and Leaf, A. The relationship of the renal excretion of adrenal corticoids to variations in renal hemodynamics. J. clin. Invest. 1953, **32**, 813.
35. Selkurt, E. E. Sodium excretion by the mammalian kidney. Physiol. Rev. 1954, **34**, 287.
36. Rosenbaum, J. D., Papper, S., and Ashley, M. M. Variations in renal excretion of sodium independent of change in adrenocortical hormone dosage in patients with Addison's disease. J. Clin. Endocr. 1955, **15**, 1459.
37. Berliner, R. W., and Davidson, D. G. Production of hypertonic urine in the absence of pituitary antidiuretic hormone. J. clin. Invest. 1956, **35**, 690.
38. Leaf, A., and Mamby, A. R. An antidiuretic mechanism not regulated by extracellular fluid tonicity. J. clin. Invest. 1952, **31**, 60.
39. Verney, E. B. Croonian lecture: The antidiuretic hormone and the factors which determine its release. Proc. roy. Soc. B 1947, **135**, 25.
40. Liddle, G. W., Duncan, L. E., Jr., and Bartter, F. C. Dual mechanism regulating adrenocortical function in man. Amer. J. Med. 1956, **21**, 380.
41. Farrell, G. L., Rosnagle, R. S., and Rauschkolb, E. W. Increased aldosterone secretion in response to blood loss. Circ. Res. 1956, **4**, 606.
42. Laragh, J. H., and Stoerk, H. C. On the mechanism of secretion of the sodium-retaining hormone (aldosterone) within the body (abstract). J. clin. Invest. 1955, **34**, 913.
43. Danowski, T. S., Elkinton, J. R., and Winkler, A. W. Movements of body water in response to acute blood loss. Amer. J. Physiol. 1946, **147**, 306.
44. Welt, L. G., and Orloff, J. The effects of an increase in plasma volume on the metabolism and excretion of water and electrolytes by normal subjects. J. clin. Invest. 1951, **30**, 751.
45. Lusk, J. A., Viar, W. N., and Harrison, T. R. Further studies on the effects of changes in the distribution of extracellular fluid on sodium excretion: Observations following compression of the legs. Circulation 1952, **6**, 911.

RELATIONSHIPS OF PYRUVATE AND LACTATE DURING ANAEROBIC METABOLISM. I. EFFECTS OF INFUSION OF PYRUVATE OR GLUCOSE AND OF HYPERVENTILATION [1]

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When the supply of oxygen to the interior of living cells is reduced to a rate insufficient for their current metabolic needs for oxygen, then various cellular oxidation-reduction systems must shift toward a more reduced state. Thus, as the oxygen tension falls the "oxidation potential" ("E_h") decreases (1, 2), (the rate of decrease being a complex and variable function of Po_2 and also of the rate of energy utilization), and the various redox systems shift toward their reduced forms in a sequence determined by their inherent redox characteristics (1), cytochrome oxidase, the cytochromes and their dependent systems, flavoproteins and diphosphopyridine nucleotide (DPN). The lowest potential in the "carrier" system is that of DPN, which is the final electron donator and functions also in the "metabolic" oxidative systems; the rates of the oxidations of energy metabolism are not affected until DPN is affected.

When the oxidative potential in the hypoxic cell has diminished to the level of the potentials of the "metabolic" systems, the first of these DPN-coupled systems to become reversed will be the one of potential closest to that of DPN: $DPNH_2$, the lactic dehydrogenase (LDH) system.

$$\textit{1)}\quad \text{Pyruvate} + DPNH_2 \overset{LDH}{\rightleftharpoons} \text{Lactate} + DPN$$

The fact that this system should shift toward the reduced state at the same time as the final transport system (DPN) appears to be an important arrangement of potentials from the point of view of the continuance of cell life during hypoxia. This is because the end-product of reduction in the LDH system is lactate, which has no other function in metabolism. All the other metabolic redox systems operate in series, so that the products of one system become the substrates of another, or several other, systems; if oxidation in one of these systems were to stop or slow down, the whole process of energy metabolism would be affected. The LDH system, however, is unique in being "dead-end;" endogenous lactate does not participate in any other equilibrium or enter into any other reaction which would be affected by its accumulation in the cell.[2]

Lactate is not the only product of a shift to the right in the steady state depicted by equation *1*). Oxidized DPN also is produced from reduced DPN. Under conditions of true oxygen deficiency within the cell, then, as the oxidation of $DPNH_2$ by molecular oxygen through the carrier system falls off, oxidation of $DPNH_2$ by the LDH system takes up. If this substitution for oxygen could occur at a sufficient rate, the oxidative potential of the cell medium would fall no further, and the remainder of the oxidations of energy metabolism could therefore continue, and life would be sustained. If the systems at any other level of potential had a significant poising effect, the fall of "E_h" might be checked (1), but in those cells which have been studied this usually does not appear to be the case (1, 2), "E_h" falling to about −0.180, that of LDH.

It is important to emphasize that the situations envisaged by this reasoning do not include *1*) complete, or even nearly complete, absence of oxygen (which would halt the succinic system), or *2*) the possible toxic effects of unlimited lactate and acid accumulation. It is true that most mammalian tissues cease to function or die in the total absence of oxygen. However, the survival of animals and human patients during a great variety of lesser hypoxic states, especially during cardiopulmonary

[1] Aided in part by a grant from the American Heart Association.

[2] This assertion is not dependent solely on the failure to discover any other lactate reaction in mammalian tissues up to the present time (3), but is confirmed by the close parallelism of specific activities of lactate and pyruvate from living tissue following exhibition of isotope-tagged pyruvate (4, 5).

disease, may be attributable to the existence of "anaerobic metabolism" such as that described above. It has been known for many years that lactate is produced in hypoxic states (6, 7) in intact animals.

If the rate at which this anaerobic metabolism occurs in intact animals during a particular period could be estimated, it would be possible to deduce in essential metabolic terms the extent to which oxygen supply was inadequate to meet tissue demands. Hill, Long, and Lupton (8), Jervell (9), and others have attempted to make such estimates from the total amount of lactate produced. However, only after their work was published was the LDH system described as an equilibrium as shown in equation *1*). It is obvious from this equation that it is not theoretically possible to use lactate alone for estimating changes in DPN : $DPNH_2$ unless pyruvate could be assumed to remain unchanged at all times. Otherwise, one of the four factors in the system will have been omitted from consideration, and some inexplicable changes in lactate production might be expected to occur. It is not surprising, therefore, to find that a number of conditions other than the state of oxidation of DPN can lead to lactate production in the absence of hypoxia (10–13). Such nonhypoxic lactate production precludes the use of lactate alone as a quantitative estimate of the anaerobic metabolism brought on by hypoxia.

If all the elements of equation *1*) are properly used, on the other hand, an exact understanding of the relationship between metabolism and oxygen supply should be possible. If equation *1*) is written in its mass action form, the following expression is obtained:

$$2) \quad [\text{Lactate}] = [\text{Pyruvate}] \times K\frac{[DPNH_2]}{[DPN]}$$

This form clearly illustrates the dependence of lactate production upon two separate factors: pyruvate and $K\frac{[DPNH_2]}{[DPN]}$, or the adequacy of oxygen supply. It may, therefore, be suggested on a theoretical basis that all instances of lactate production by tissues can be divided into three types: lactate production due to (*a*) inadequacy of oxygen supply relative to metabolic needs, *i.e.*, change in the factor $K\frac{[DPNH_2]}{[DPN]}$ alone; (*b*) change in pyruvate production alone; and (*c*) changes in both factors simultaneously.

For all practical purposes (*a*) does not seem to occur, in our experience, in dogs, goats or human patients, although an occasional experiment may approximate it, presumably merely as one form of group (*c*). It becomes important, however, to distinguish lactate changes due to (*b*) from those due to (*c*).

This has been done by studying a tissue or an animal at two points in time with respect to the quantities of pyruvate and lactate present. An expected lactate value for an experimental period t_n is calculated from the figure for $K\frac{[DPNH_2]}{[DPN]}$ obtained at a control time, t_o, plus the observed pyruvate change, $P_n - P_o$. In other words, it is initially assumed for the purpose of determining the theoretical change in lactate resulting only from the change in pyruvate that no hypoxia occurred between t_o and t_n. If the amount of change in lactate actually observed is equal to this, then it is concluded that all the lactate produced is accounted for simply by the change in pyruvate. If the amount of change in lactate actually observed exceeds this predicted value, $\widehat{\Delta L}$, then the assumption of no hypoxia was incorrect; but the "excess lactate" obtained by difference $[(L_n - L_o) - \widehat{\Delta L}]$ can be present only because a change in $K\frac{[DPNH_2]}{[DPN]}$ has occurred, and serves as a measure of this change. The final equation for calculating this theoretical fraction of the total concentration change, the "excess lactate" or XL, is derived in the appendix and is as follows:

$$3) \quad XL = (L_n - L_o) - (P_n - P_o)(L_o/P_o)$$

where L_n and L_o are experimental and control lactate concentrations, P_n and P_o experimental and control concentrations of pyruvate, respectively.

In order to examine the pertinence of this theoretical quantity, XL, to body physiology, human subjects and animals were subjected to a group of conditions which were known to produce lactate accumulation without, insofar as it can be determined, any effect on oxygen supply. The simplest and most direct of these was infusion of pyruvate. Infusion of glucose represents a similar stimulus but one which more nearly imitates physiologic changes which may take place

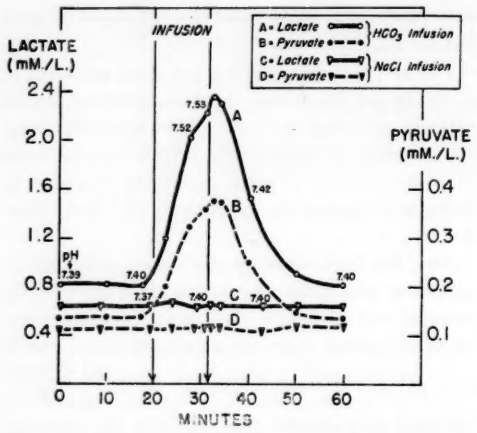

FIG. 1. TYPICAL CHANGES IN BLOOD LACTATE AND PYRUVATE IN A DOG DURING INTRAVENOUS INFUSION OF SODIUM BICARBONATE SOLUTION (CURVES A AND B) AND SODIUM CHLORIDE SOLUTION (CURVES C AND D)

The effect of bicarbonate is essentially the same as that of hyperventilation or the infusion of sodium pyruvate. No change occurred in arterial or mixed venous blood oxygen saturations. Excess lactate values zero.

naturally. The third, hyperventilation and alkalosis, was studied both because it produces the most striking lactate accumulations and because pH changes are complicating factors in most studies of hypoxia.

METHODS

Blood samples from human subjects were collected as previously described (14). Briefly, an indwelling arterial needle was inserted into a brachial artery under local anesthesia, and blood was allowed to flow rapidly through a short length of plastic tubing directly into a weighed tube of ice cold 10 per cent trichloracetic acid in 0.5 N HCl. Lactate and pyruvate analyses were carried out in triplicate as previously described (14); each recorded figure represents the mean of all analyses on two blood samples collected in rapid succession. Reproducibility of successively drawn samples during a steady state was about 0.5 per cent for lactate and 1.0 per cent for pyruvate. Blood samples were collected from dogs in the same manner except that each arterial sample was accompanied by a similarly collected sample of mixed venous blood from a wide-bore brass cannula inserted through the jugular vein into the right ventricle of the heart. Blood water content was determined with each sample and all concentrations are given in millimoles per liter of blood water. Blood oxygen determinations were carried out either manometrically in the Van Slyke apparatus, or spectrophotometrically (15). The pH of blood samples was determined with a glass electrode at 37° C. after collection in sodium fluoride and preservation in ice for about an hour.

Oxygen consumption rates were calculated from the minute volume of inspired air delivered from a spirometer and the oxygen, carbon dioxide, and nitrogen contents of expired air as determined with the standard Haldane apparatus. Control samples were taken after an hour of rest. Animals were anesthetized with 20 mg. chloralose per Kg., although the present results have been duplicated in animals under pentobarbital anesthesia when arterial oxygen saturation was normal.

RESULTS

Infusing sodium pyruvate solutions having pH 7.0 or 7.4 led to a considerable alkalinizing effect. Such infusions had previously been given by Bueding and Goldfarb (16) and tacitly compared, presumably, with control infusions of sodium chloride. The proper control infusate, however, should be sodium bicarbonate solution (17, 18) which has an effect, as shown in Figure 1 (curves A and B), essentially identical with that of sodium pyruvate. From this observation it might be concluded that pyruvate infusion, in fact, has no specific effect on blood lactate, but only the effect of an alkalinizing salt. The same effects on blood pyruvate and pH were elicited with 0.10 M sodium pyruvate solutions at infusion rates between 1 and 4 ml. per minute (0.2 to 0.4 mM per minute) for

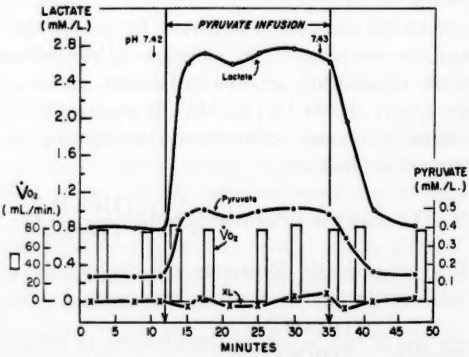

FIG. 2. TYPICAL EFFECTS OF INFUSION OF BUFFERED 0.5 M PYRUVATE SOLUTION (pH 5.5) ON BLOOD LACTATE AND PYRUVATE AND ON RATE OF OXYGEN CONSUMPTION IN AN ANESTHETIZED DOG

Absence of change in oxygen consumption and on oxygen saturation of arterial (96.2 per cent) and right ventricular blood (70.9 per cent) gave no evidence of hypoxia.

TABLE I

Effect of intravenous infusion of acidic pyruvate solutions in dogs on blood lactate and pyruvate concentrations *

Procedure	Lactate	Pyruvate	XL†	pH	$\dot{V}_{O_2}$†	$S\bar{v}_{O_2}$†
	mM/L.	*mM/L.*	*mM/L.*		*ml./min.*	%
Control	0.695	0.120	0	7.41	66	74
Infusion	3.788	0.648	0.033	7.40	65	76
Recovery	0.709	0.121	0.008	7.40	65	73
Control	0.722	0.143	0	7.39	88	72
Infusion	2.090	0.410	0.023	7.41	89	75
Recovery	0.730	0.144	0.003	7.41	90	75
Control	0.652	0.125	0	7.40	92	75
Infusion	4.920	0.946	0	7.40	90	77
Recovery	0.700	0.133	0.006	7.38	90	74
Control	0.820	0.128	0	7.42	71	71
Infusion	2.876	0.453	0	7.39	74	74
Recovery	0.815	0.127	0	7.42	73	71
Control	0.871	0.142	0	7.38	102	76
Infusion	3.720	0.600	0.039	7.40	102	78
Recovery	0.860	0.138	0.014	7.39	100	74

* Only experiments with unaltered blood pH are shown. Values marked "infusion" were obtained 5 minutes after stopping the infusion; "recovery" values were obtained 30 minutes later.

† XL = Excess lactate.
$\dot{V}_{O_2}$ = Oxygen consumption.
$S\bar{v}_{O_2}$ = Mixed venous blood oxygen saturation.

TABLE II

Arterial blood lactate and pyruvate concentrations 15 minutes after intravenous infusions of 10 per cent glucose solutions for 30 minutes in human subjects, and dogs anesthetized with chloralose, showing no calculated excess lactate formation (XL)

Subject Procedure	Lactate	Pyruvate	XL
	mM/L.	*mM/L.*	*mM/L.*
Human			
Control	0.542	0.132	0
Glucose, I.V.	0.655	0.157	0.010
Human			
Control	0.481	0.125	0
Glucose, I.V.	0.580	0.149	0.006
Human			
Control	0.511	0.127	0
Glucose, I.V.	0.699	0.170	0.016
Human			
Control	0.420	0.119	0
Glucose, I.V.	0.510	0.144	0
Dog			
Control	0.924	0.131	0
Glucose, I.V.	1.357	0.191	0.013
Dog			
Control	0.775	0.123	0
Glucose, I.V.	1.248	0.194	0.023
Dog			
Control	0.784	0.119	0
Glucose, I.V.	1.154	0.176	0

periods of time from 5 to 20 minutes. The sudden single injections of very large quantities, as previously done (16), were considered difficult to interpret, so that continuous infusions were em-

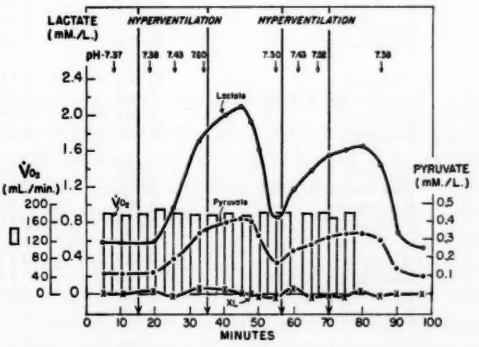

FIG. 3. TYPICAL CHANGES IN OXYGEN CONSUMPTION AND IN BLOOD LACTATE, PYRUVATE, AND pH IN A HUMAN SUBJECT DURING TWO PERIODS OF VOLUNTARY HYPERVENTILATION

Ventilatory volume was increased 4.2- and 3.4-fold, respectively. There was no significant change in oxygen consumption or in brachial arterial oxygen saturation (95.7 per cent to 96.9 per cent) or pulmonary arterial saturation (70.2 per cent to 72.6 per cent) during the procedure to suggest the presence of hypoxia. Values of excess lactate for each sampling time lie on the zero line, the maximum value being 0.033 mM per L.

ployed instead. It subsequently proved to be possible to give continuous intravenous infusions of pyruvate to dogs without altering blood pH. The infusates consisted of 0.5 molar pyruvic acid buffered at pH between 5.0 and 6.0 When this was done, neither blood lactate nor pyruvate rose during infusions at the previous rates. However, when larger amounts were given [rates between 2 and 5 ml. per minute (0.5 to 2.5 mM per minute)], the great capacity of the body to absorb pyruvate apparently could be overloaded and blood pyruvate made to rise. The experiments in which this was accomplished without change in blood pH are shown in Figure 2 and Table I. It was found that tissue lactate production followed the rise in body pyruvate. Rates of oxygen consumption remained constant if blood pH remained constant; mixed venous blood oxygen saturation did not fall. Hypoxia of the severity which might lead to these large lactate accumulations, therefore, did not appear to be present. The blood pyruvate levels fell promptly following cessation of pyruvate infusion.

Table II shows the effects of infusion of 500 ml. of 10 per cent glucose into human subjects in 30 minutes. The animals received a similar amount (1 Gm. per Kg. body weight) in 30 minutes. The results are in agreement with those previously published (19); both lactate and pyruvate concentrations rose, and the ratio of the two changed only slightly.

Figure 3 and Table III show the increase in blood lactate which resulted from voluntary hyperventilation in human subjects. From the measurements of rate of oxygen consumption ($\dot{V}_{O_2}$) it is clear that no measurable reduction occurred in the rate of delivery of oxygen per minute to the tissues, and that no O_2-debt was contracted. In two instances mixed venous blood was sampled from the pulmonary artery during the hyperven-

TABLE III

Effect of voluntary hyperventilation for 15 minutes in human subjects *

Procedure	Lactate	Pyruvate	XL	pH	$\dot{V}_{O_2}$	$S\bar{v}_{O_2}$
	mM/L.	*mM/L.*			*ml./min.*	%
1) Control	0.696	0.135	0	7.40	252	74
Ventilation × 3.49	0.999	0.195	0	7.47	280	77
Recovery 1	1.799	0.343	0.029	7.43	250	75
Recovery 2	0.675	0.130	0	7.41	255	73
2) Control	0.772	0.131	0	7.39	287	72
Ventilation × 4.60	1.241	0.207	0.021	7.53	316	78
Recovery 1	2.099	0.347	0.052	7.40	280	72
Recovery 2	0.683	0.116	0	7.40	295	74
3) Control	0.582	0.124	0	7.42	312	
Ventilation × 3.05	0.643	0.137	0	7.50	327	
Recovery 1	0.991	0.204	0.033	7.41	310	
Recovery 2	0.570	0.143	0	7.40	330	
4) Control	0.629	0.125	0	7.40	195	
Ventilation × 4.01	0.940	0.182	0.025	7.51	220	
Recovery 1	1.877	0.361	0.063	7.42	200	
Recovery 2	0.650	0.126	0.016	7.40	204	
5) Control	0.560	0.132	0	7.41	251	
5% CO_2, Vent. × 4.20	0.565	0.129	0.018	7.39	267	
Recovery 1	0.559	0.131	0	7.41	242	
Recovery 2	0.563	0.132	0	7.41	242	
6) Control	0.607	0.142	0	7.40	275	
5% CO_2, Vent. × 3.56	0.608	0.145	0	7.40	296	
Recovery 1	0.620	0.140	0.021	7.41	270	
Recovery 2	0.615	0.141	0.016	7.40	275	

* Arterial blood lactate, pyruvate, pH, oxygen consumption ($\dot{V}_{O_2}$) and mixed venous blood oxygen saturation ($S\bar{v}_{O_2}$) were measured, and excess lactate (XL) calculated as described. Two additional hyperventilation periods are given in Figure 1. In Experiments 5 and 6 hyperventilation was induced with 5 per cent CO_2 in 20 per cent O_2, 75 per cent N_2. Values marked "hyperventilation" were obtained at the end of the forced breathing period; "Recovery 1" is the peak value of pyruvate; "Recovery 2" values obtained 45 minutes after cessation of hyperventilation.

TABLE IV

Effect of threefold increase in pump-controlled pulmonary ventilation in dogs upon blood lactate and pyruvate concentrations

Procedure	Lactate	Pyruvate	XL*	pH	$\dot{V}_{O_2}$*	$S\bar{v}_{O_2}$*
	mM/L.	*mM/L.*			*ml./min.*	%
1) Control	0.724	0.120	0	7.41	60	75
Hyperventilation	2.460	0.402	0.032	7.67	67	82
Recovery 1	3.657	0.590	0.093	7.39	62	77
Recovery 2	0.680	0.113	0	7.40	64	75
2) Control	0.655	0.129	0	7.38	96	74
Hyperventilation	1.840	0.361	0.006	7.49	104	79
Recovery 1	2.789	0.542	0.034	7.40	93	75
Recovery 2	0.650	0.127	0.005	7.39	95	75
3) Control	0.789	0.127	0	7.36	71	70
Hyperventilation	1.092	0.175	0.004	7.52	79	77
Recovery 1	2.009	0.324	0.005	7.38	70	77
Recovery 2	0.776	0.125	0	7.36	70	72
4) Control	0.851	0.133	0	7.41	67	75
Hypervent.—100% O_2	2.052	0.318	0.016	7.54	74	90
Recovery 1	3.657	0.560	0.076	7.41	68	78
Recovery 2	0.857	0.133	0	7.41	69	76
5) Control	0.898	0.112	0	7.42	98	72
Hypervent.—100% O_2	2.751	0.337	0.048	7.59	107	89
Recovery 1	3.421	0.412	0.118	7.41	98	80
Recovery 2	0.901	0.111	0.011	7.41	96	74
6) Control	0.750	0.139	0	7.38	82	74
Hypervent.—5% CO_2	0.720	0.131	0	7.36	82	87
Recovery 1	0.729	0.133	0	7.38	82	80
Recovery 2	0.745	0.137	0	7.39	83	75
7) Control	0.689	0.141	0	7.42	109	71
Hypervent.—95% O_2, 5% CO_2	0.605	0.122	0	7.40	111	91
Recovery 1	0.670	0.135	0	7.43	113	80
Recovery 2	0.662	0.133	0	7.43	113	76

* See Table I for identification of these abbreviations.

tilation; the oxygen saturations show no decrease in the mean Po_2 of blood draining body tissues, and cardiac output was not diminished. Although it, therefore, seems very unlikely that hypoxia was produced by the hyperventilation, lactate production was, nevertheless, very prominent. An increase in muscular exertion occurred with voluntary hyperventilation, however, and this may account for the lactate production.

Table IV, on the other hand, shows that similar results were obtained in dogs which were passively hyperventilated by means of a respiratory pump; in each instance minute volume of respiration was increased threefold over the normal resting value by first doubling tidal volume and then adjusting the respiratory rate, during repeated collections of expired air. Again no auxiliary evidence of oxygen deficiency could be found to account for the lactate production. Indeed, improved oxygenation, as in Experiments 4 and 5, failed to prevent the lactic acidemia. If blood pH was prevented from rising, however, by inclusion of 5 per cent carbon dioxide in the gas mixture inspired, lactate accumulation failed to occur during the hyperventilation as shown in Experiments 6 and 7 of Table III and Experiments 6 and 7 of Table IV. The rate of lactate accumulation showed no clear quantitative relationship to the amount of rise of blood pH in these experiments.

The maximum value of blood pyruvate was reached in all experiments after the end of hyperventilation when normal breathing had resumed. The time of the peak varied from 8 to 28 minutes following the resumption of normal breathing, with a mean time of 14 minutes. Timing of the lactate changes was the same.

The outstanding correlation revealed in these data is that between blood concentrations of lactate and of pyruvate. The amount of change in pyruvate was, of course, quite different from that

in lactate concentration, but the two bore a consistent straight line relationship during their alterations (in any one individual) so that the ratio changed little.

Methods of collecting, preserving and analysing blood for lactate and pyruvate have varied considerably in the past. It is difficult to rule out the possibility that the methods used have greatly affected some of the results published, and these should be considered in detail.

The present techniques have yielded the following basal concentrations in arterial blood of 69 normal human subjects: lactate, 0.618 mM per L. blood H_2O ($\sigma = 0.077$); pyruvate, 0.142 mM per L. blood H_2O ($\sigma = 0.026$); lactate-pyruvate ratio, 4.24 ($\sigma = 0.44$). These values differ considerably from those in previous reports.

DISCUSSION

Mean resting human blood pyruvate values of 0.045 to 0.085 mM per L. (20), 0.088 (21), 0.116 (19), and 0.114 (22) have been reported. Peripheral venous blood was used, with varying degrees of care in rapidity of denaturation. Bueding and Wortis (23) believed pyruvate decreased rapidly in shed blood, changes being significant within a few seconds. We have confirmed this finding (14).

Corresponding values for blood lactate have been reported to be 1.11 mM per L. (13), 1.45 (24), 1.54 (25), and 1.13 mM per L. (21). After the development of the precise and highly specific analytical method of Barker and Summerson (26), mean values of 0.887 mM per L. (27), 1.03 (22), and 0.667 mM per L. (28) were reported.

Mean resting lactate-pyruvate ratios have been 13.4 (2), 13.2 (21), 9.3 (19), 11.3 (21), and 10.6 (28). It has been stated that the "basal state" does not give the lowest lactate-pyruvate ratio and that fairly vigorous walking produces no increase in it (19), or in lactate (13).

In the present study, using arterial blood and no lapse of time between sampling and denaturation of the blood, quite different conclusions have been arived at. Measurements by these techniques have shown arterial blood lactate and lactate-pyruvate ratio to increase immediately (in less than one minute) on the mildest exertion (29), although analysis of venous blood does not reveal this change. Our data on the resting state indicate that bed rest and the nearest possible approach to basal conditions yields the lowest body lactate and lactate-pyruvate ratio, and these minimal reference values are much lower than previously reported. Lactate, pyruvate, and the lactate-pyruvate ratio always fell during the resting control periods of one and one-half to two and one-half hours, and a plateau was usually reached in one hour. The average fall in lactate concentration in 32 human subjects was 0.320 mM per L. H_2O, of pyruvate, 0.021 mM per L., and of the lactate-pyruvate ratio, 2.60.

It has been shown that any delay in the denaturation of blood causes much lower pyruvate and somewhat higher lactate concentrations, with very significantly higher ratios of lactate to pyruvate than appear to exist in the body (14). We have found that fluoride significantly accelerates the immediate disappearance of pyruvate from shed blood and the rise of the lactate-pyruvate ratio. Iodoacetate produces a rise in blood pyruvate with time *in vitro* and, therefore, an artifactual depression of the lactate-pyruvate ratio. The greatest difference in results, however, is produced by the use of peripheral venous blood, an error which has been pointed out previously (30–32). It must be recognized that these values may reflect purely local events in the limb as much as they reflect changes in total body lactate and pyruvate. During changing levels the error may be 100 per cent or more. In 15 of our normal basal subjects, blood drawn from a vein by the method of Friedemann, Haugen, and Kmieciak (21) gave a mean lactate concentration of 1.222 mM per L. blood H_2O ($\sigma = 0.354$), as compared with a simultaneous value of 0.643 ($\sigma = 0.079$) by the present method, and a mean pyruvate concentration of 0.119 mM per L. ($\sigma = 0.049$), as compared with 0.146 ($\sigma = 0.026$).

The present data were obtained with these technical improvements and appear to demonstrate a rather marked lactate production in the body in response to slight changes in body pH, glucose and pyruvate in the complete absence of oxygen lack. They also suggest the mechanism for this occurrence, as outlined in the introduction. One of the two factors affecting the body lactate content is the tissue oxygen deficiency factor; the other, however, is pyruvate concentration, which may change considerably in the absence of any oxygen

lack, as illustrated in the present data. This is demonstrated most simply by changing pyruvate artificially by infusing this substance. One of the most potent gross stimuli to endogenous pyruvate production in the intact body, however, appears to be a change in acid-base balance. Since pyruvate levels are the resultant of a great number of different chemical equilibria in the body, it is not possible to quantitate the various causes for pyruvate change during hypocapnia. Whatever may be the detailed reasons, when pyruvate does change it is possible to predict from equation *2*) that lactate will change also, and that it will change by an amount which can be derived from the equation (if hypoxia is absent). Equation *2*), of course, states that the ratio of lactate to pyruvate should remain constant during the interval under study if hypoxia is absent. Some change in the lactate-pyruvate ratio did occur in the present experiments, although it seems very small.

Only if no change in the lactate-pyruvate ratio had occurred could the significance mentioned be assigned to this observation. If some change did occur, it would then be impossible to know in any real terms how significant this might be. One could only say, in that case, that the equilibrium of equation *1*) was being displaced to the right to some unknown extent. The total rate of this shift might be very great for even such a small distortion of the lactate-pyruvate ratio as seen in the present experiments, or it might be negligible. Rates of change of "excess lactate" concentrations in all the experiments reported here, when multiplied by the volume of total body water and by a factor of 11.2 to convert millimoles to oxygen equivalents (*29*), constitute less than 0.1 per cent of the rate of oxygen consumption. This appears to be negligible, and the essential constancy of $K\frac{[DPNH_2]}{[DPN]}$ in the present experiments is reliably confirmed. Under other conditions the "excess lactate" has proved to be of very significant proportions relative to oxygen consumption, but this situation is physiologically quite different and is dealt with separately (*29*).

The constancy of these relationships in the present experiments has some important implications with respect to metabolic studies generally in man and intact animals, when blood analysis must be employed. The lactic dehydrogenase system lies within the cells, but concentrations were measured in these subjects in the blood. In the alkalinization experiments, therefore, the arterial blood lactate concentrations appear to have changed in a manner which accurately reflected the mean of theoretical intracellular changes in the body as calculated above. Passive diffusion across cell membranes (although not identical concentrations in intracellular and extracellular fluids) has previously been proposed for these ions (14, 33). In the pyruvate infusion experiments, moreover, the primary pyruvate change occurred in the blood and must have effected secondarily a change within the cells which then altered lactate concentration, first intracellularly and subsequently in the blood. Despite this double transit across cell membranes, blood lactate still followed blood pyruvate rather faithfully.[3] Such small amounts of calculated "excess lactate" as were present may represent lags in diffusion equilibration.

Lactate production theoretically is not controlled exclusively by the adequacy of cellular oxygenation, and is demonstrably affected to a very significant extent by the pyruvate changes of overventilation or pH alterations of the body, of blood glucose changes and probably other stimuli. It would, therefore, seem quite inadvisable to draw any conclusions about tissue oxygen supply from determinations of lactate alone. Hyperventilation in human subjects has previously been noted to be associated with lactic acidemia (13, 17), but muscular exertion also occurred, and no clear explanation for the finding was apparent. Similar effects of pH on tissue lactate production may be deduced from data reported in the heart-lung preparation (34) in isolated limb preparations (35), and in intact dogs (18, 36). Our findings augment these data and suggest that the changes in lactate may be interpreted as responses to pyruvate accumulation.

The pH effect, however, is only one of several general conditions leading to lactate accumulation without hypoxia. Himwich (10) reviewed the effect of administering various carbohydrates in

[3] Even in pieces of excised tissue, devoid of circulation altogether, a double transit path in the same sense appears to lead to only about 15 per cent difference in specific activity of lactate and isotope-tagged pyruvate in the incubation medium (4) and close parallelism during changing activity (5).

producing lactic acidemia. Rises in blood lactate in human subjects due to glucose administration have been studied more recently by Bueding, Stein, Wortis, and Goldfarb (11, 16) and many others. The work cited likewise demonstrated a pyruvic acid accumulation of corresponding magnitude resulting from glucose injection.

Epinephrine causes increased lactate production by muscle (10) which is not due to diminished blood flow (37) or altered arteriovenous oxygen difference (38), and produces lactate accumulation (12). Again, pyruvate accumulation is known to occur as a result of epinephrine injection (39).

In view of the significant changes in carbohydrate metabolism resulting from pH change (40, 41), it appears possible as an interrelation of the diverse events listed above that all the stimuli mentioned produce pyruvate accumulation through effects on the processes of carbohydrate metabolism which they have in common.

However this may be, it is suggested from the present studies that lactate accumulation ensues as a result of its tendency to maintain equilibrium with the pyruvate present, and the equations derived for this relationship appear to suggest that experimental lactate changes fit the concept satisfactorily. With further investigation such a concept might help to clarify the complex relationship between tissue oxygen deficiency and lactic acid production.

Considering the rapidity with which lactic acid accumulation may be brought about, this phenomenon observed during hyperventilation represents a mechanism for quickly reducing body bicarbonate as an adjustment to alkalosis. We have seen several human subjects with pathological hyperventilation who replaced half or more of their serum bicarbonate with lactate in the course of several hours. Considering the fact that 10 to 12 mM per L. of lactic acid may at times be found to accumulate within a few minutes, this mechanism of acid-base homeostasis can have almost as great rapidity of response as carbon dioxide tension itself. If a patient's primary derangement is respiratory alkalosis, the LDH mechanism of tissue acid production would appear to be the only immediate compensatory response available.

SUMMARY AND CONCLUSIONS

1. Small and large accumulations of lactate were produced in human subjects and animals by methods which would not be expected to induce hypoxia: hyperventilation and infusion of bicarbonate, pyruvate or glucose. Arterial and mixed venous blood oxygen saturations and rates of oxygen consumption gave no indication that any detectable hypoxia was induced by the procedures.

2. Simultaneous accumulations of pyruvate occurred in each instance.

3. The lactate changes were found to correspond closely to those of pyruvate according to equations derived for steady state of the lactic dehydrogenase system and absence of hypoxia.

4. It is concluded that production of lactate by tissues in intact man and animals has no necessary significance with respect to hypoxia of the tissues.

5. A calculated fraction of any total lactate change ("excess lactate") is theoretically not subject to the error of nonhypoxic production and appears experimentally to be essentially zero throughout wide fluctuations of total lactate induced by nonhypoxic stimuli.

6. The data suggest that changes in blood concentrations of pyruvate and lactate in intact man and animals, when estimated by the methods described, closely approximate those occurring at the loci of the enzyme lactic dehydrogenase, *i.e.*, within tissue cells.

APPENDIX

The lactic dehydrogenase system may be depicted by:

1) $$C_3H_4O_3 + DPNHH^+ \rightleftharpoons C_3H_5O_3 + DPN$$

or by the mass action form:

2) $$[\text{Pyruvate}] \times [DPNHH^+] = K[\text{Lactate}] \times [DPN]$$

which may be rearranged to show the two physiologic factors affecting lactate concentration at any time t_o:

3) $$[\text{Lactate}]_o = [\text{Pyruvate}]_o \times K\frac{[DPNH^+H]_o}{[DPN]_o}$$

The difference in lactate between t_o and any other time t_n, provided there is no hypoxia, will be:

4) $$[\text{Lactate}]_n - [\text{Lactate}]_o = [\text{Pyruvate}]_n - [\text{Pyruvate}]_o \times K\frac{[DPNH_2]_o}{[DPN]_o}$$

Substituting from equation *3)* the value obtained by solving for $K\frac{[DPNH_2]_o}{[DPN]_o}$:

5) $$[\text{Lactate}]_n - [\text{Lactate}]_o = [\text{Pyruvate}]_n - [\text{Pyruvate}]_o \times \frac{[\text{Lactate}]_o}{[\text{Pyruvate}]_o}$$

The difference between the value of $\hat{L}_n - \hat{L}_o$, predicted from equation *5)* on the assumption that no true hypoxia

occurred within the cells, and the value of $L_n - L_o$ actually observed to occur is the "excess lactate:"

6) $$XL = \Delta L - \widehat{\Delta L}$$

and in terms of measurable quantities (all concentrations):

7) $$XL = (L_n - L_o) - (P_n - P_o)(L_o/P_o)$$

A sample calculation gives the following result:

Basal blood lactate = 0.550 mM per L.
Basal blood pyruvate = 0.135 mM per L.
Experimental lactate value = 2.680 mM per L.
Experimental pyruvate value = 0.409 mM per L.

$$XL = (2.680 - 0.550) - (0.409 - 0.135)\left(\frac{0.550}{0.135}\right)$$
$$= 2.130 - 1.115$$
$$XL = 1.015 \text{ mM per L.}$$

REFERENCES

1. Hewitt, L. F. Oxidation-reduction potentials *in* Bacteriology and Biochemistry, 6th ed. Baltimore, Williams and Wilkins Co., 1950, pp. 95–126.
2. Baumberger, J. P. The relation between the "oxidation-reduction potential" and the oxygen consumption rate of yeast cell suspensions. Cold Spr. Harb. Symp. quant. Biol. 1939, **7**, 195.
3. Stotz, E. Pyruvate metabolism. Advanc. Enzymol. 1945, **5**, 129.
4. Teng, C., Karnovsky, M. L., Landau, B. R., Hastings, A. B., and Nesbett, F. B. Metabolism of C^{14}-labeled glycerol and pyruvate by liver in vitro. J. biol. Chem. 1953, **202**, 705.
5. Foster, J. M., and Villee, C. A. Pyruvate and acetate metabolism in the isolated rat diaphragm. J. biol. Chem. 1954, **211**, 797.
6. Araki, T. Ueber die Bildung von Milchsäure und Glycose im Organismus bei Sauerstoffmangel. Hoppe-Seylers Z. physiol. Chem. 1890, **15**, 335.
7. Hastings, A. B. The lactic acid in the blood of dogs in exercise. Proc. Soc. exp. Biol. (N. Y.) 1920–1921, **18**, 306.
8. Hill, A. V., Long, C. N. H., and Lupton, H. Muscular exercise, lactic acid and the supply and utilization of oxygen. V. The recovery process after exercise in man. Proc. roy. Soc. B. 1924, **97**, 96.
9. Jervell, O. Investigation of the concentration of lactic acid in blood and urine under physiologic and pathologic conditions. Acta. med scand. 1928, Suppl. **24**, 1.
10. Himwich, H. E. The role of lactic acid in the living organism. Yale J. Biol. Med. 1931–1932, **4**, 259.
11. Bueding, E., Stein, M. H., and Wortis, H. Blood pyruvate curves following glucose ingestion in normal and thiamine-deficient subjects. J. biol. Chem. 1941, **140**, 697.
12. Cori, C. F., and Cori, G. T. Mechanism of epinephrine action. IV. The influence of epinephrine on lactic acid production and blood sugar utilization. J. biol. Chem. 1929, **84**, 683.
13. Bock, A. V., Dill, D. B., and Edwards, H. T. Lactic acid in the blood of resting man. J. clin. Invest. 1932, **11**, 775.
14. Huckabee, W. E. Control of concentration gradients of pyruvate and lactate across cell membranes in blood. J. appl. Physiol. 1956, **9**, 163.
15. Huckabee, W. E. Spectrophotometric analysis of uncontaminated blood for oxyhemoglobin. J. Lab. clin. Med. 1955, **46**, 486.
16. Bueding, E., and Goldfarb, W. J. Blood changes following glucose, lactate, and pyruvate injections in man. J. biol. Chem. 1943, **147**, 33.
17. Fenn, W. O., Rahn, H., Otis, A. B., and Chadwick, L. E. Physiological observations on hyperventilation at altitude with intermittent pressure breathing by the pneumolator. J. appl. Physiol. 1948, **1**, 773.
18. Haldi, J. Lactic acid in blood and tissues following intravenous injection of sodium bicarbonate. Amer. J. Physiol. 1933, **106**, 134.
19. Bueding, E., Wortis, H., and Stern, M. Pathological variations in blood and spinal fluid pyruvic acid. J. clin. Invest. 1942, **21**, 85.
20. Platt, B. S., and Lu, G. D. Studies on the metabolism of pyruvic acid in normal and vitamin B_1-deficient states. IV. The accumulation of pyruvic acid and other carbonyl compounds in beri-beri and the effect of vitamin B_1. Biochem. J. 1939, **33**, 1525.
21. Friedemann, T. E., Haugen, G. E., and Kmieciak, T. C. Pyruvic acid. III. The level of pyruvic and lactic acids, and the lactic-pyruvate ratio, in the blood of human subjects. The effect of food, light muscular activity and anoxia at high altitude. J. biol. Chem. 1945, **157**, 673.
22. Goldsmith, G. A. The blood lactate-pyruvate relationship in various physiologic and pathologic states. Amer. J. med. Sci. 1948, **215**, 182.
23. Bueding, E., and Wortis, H. The stabilization and determination of pyruvic acid in the blood. J. biol. Chem. 1940, **133**, 585.
24. Weiss, S., and Ellis, L. B. Oxygen utilization and lactic acid production in the extremities during rest and exercise. Arch. intern. Med. 1935, **55**, 665.
25. Hallock, P. Lactic acid production during rest and after exercise in subjects with various types of heart disease with special reference to congenital heart disease. J. clin. Invest. 1939, **18**, 385.
26. Barker, S. B., and Summerson, W. H. The colorimetric determination of lactic acid in biological material. J. biol. Chem. 1941, **138**, 535.
27. Decker, D. G., and Rosenbaum, J. D. The distribution of lactic acid in human blood. Amer. J. Physiol. 1942, **138**, 7.
28. Bay, E., Barron, E. S. G., Adams, W., Case, T., Halstead, W. C., and Ricketts, H. T. The behavior of blood lactate and pyruvate with exercise at sea-level and at altitude. Part III. National Research Council Report No. 344, Committee on Aviation Medicine, 1944.

29. Huckabee, W. E. Relationships of pyruvate and lactate during anaerobic metabolism. II. Exercise and formation of O_2-debt. J. clin. Invest. 1958, **37**, 255.
30. Himwich, H. E., Loebel, R. O., and Barr, D. P. Studies of the effect of exercise in diabetes. I. Changes in acid-base equilibrium and their relation to the accumulation of lactic acid and acetone. J. biol. Chem. 1924, **59**, 265.
31. Barr, D. P., and Himwich, H. E. Studies in the physiology of muscular exercise. II. Comparison of arterial and venous blood following vigorous exercise. J. biol. Chem. 1923, **55**, 525.
32. Eggleton, M. G., and Evans, C. L. The lactic acid content of blood after muscular contraction under experimental conditions. J. Physiol. 1930, **70**, 269.
33. Newman, E. V. Distribution of lactic acid between blood and muscle of rats. Amer. J. Physiol. 1938, **122**, 359.
34. Anrep, C. V., and Cannan, R. K. The concentration of lactic acid in the blood and in experimental alkalaemia and acidaemia. J. Physiol. 1923, **58**, 244.
35. Eggleton, M. G., and Evans, C. L. Lactic acid formation and removal with change of blood reaction. J. Physiol. 1930, **70**, 261.
36. Gesell, R., Krueger, H., Gorham, G., and Bernthal, T. The regulation of respiration; a study of the correlation of numerous factors of respiratory control following administration of hydrochloric acid, of carbon dioxide, and the simultaneous administration of CO_2 and sodium bicarbonate. Amer. J. Physiol. 1930, **94**, 402.
37. Cori, C. F., Fisher, R. E., and Cori, G. T. The effect of epinephrine on arterial and venous plasma sugar and blood flow in dogs and cats. Amer. J. Physiol. 1935, **114**, 53.
38. Griffith, F. R., Jr., Omachi, A., Lockwood, J. E., and Loomis, T. A. The effect of intravenous adrenalin on blood flow, sugar retention, lactate output and respiratory metabolism of peripheral (leg) tissues in the anesthetized cat. Amer. J. Physiol. 1947, **149**, 64.
39. Flock, E., Bollman, J. L., and Mann, F. C. Utilization of pyruvic acid by the dog. J. biol. Chem. 1938, **125**, 49.
40. Haldane, J. B. S., Wigglesworth, V. B., and Woodrow, C. E. The effect of reaction changes on human carbohydrate and oxygen metabolism. Proc. roy. Soc. B. 1924, **96**, 15.
41. Guest, M. M., and Rawson, R. A. Carbohydrate storage and mobilization with changes in blood pH. J. biol. Chem. 1941, **139**, 535.

RELATIONSHIPS OF PYRUVATE AND LACTATE DURING ANAEROBIC METABOLISM. II. EXERCISE AND FORMATION OF O_2-DEBT [1]

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On the basis of previous studies, it has been suggested that lactic acid production by tissues may be regarded as a function of the effectiveness of oxygen supply to them (1–3). However, these studies are subject to certain technical objections (4), and various other investigations have appeared to show either a different relationship (5, 6) or no relationship of lactate to O_2-debt (2, 7, 8) or hypoxia (9). Furthermore, a variety of conditions unrelated to oxygen deficiency have been found to cause lactate production in the intact body (4). Therefore, it seems unwarranted at present to ascribe alterations in body lactate, or in lactate exchanges of organs, to oxygen deficiency of the tissues.

The essential element of the original suggestion by Hill, Long, and Lupton that lactate production is associated with oxygen deficiency in the intact body was an apparent relationship between lactate and O_2-debt found under very special circumstances (1, 10, 11). We have been unable to confirm this relationship strictly, as will be seen in the present data. The development of knowledge about the lactic dehydrogenase system in recent years, moreover, makes such a relationship appear to have been a special circumstance rather than a general principle, since changes in pyruvate affect lactate levels as much as does oxygen lack (4), and the fundamentally parallel effect of pyruvate and hypoxia on lactate production is shown by the equation of the lactic dehydrogenase equilibrium. If a lactate change, however, exceeds that which is appropriate, according to this relationship, to the alterations in pyruvate, then there would appear to be only one explanation for the excess, *i.e.*, oxygen deficiency. Calculations of this "excess lactate" (XL) previously made (4) show this quantity to be essentially zero during marked lactate production from nonhypoxic causes. The present data on exercise indicate the magnitude of "excess lactate" production during tissue hypoxia and the quantitative relationships between total lactate, XL and respiratory O_2-debt.

[1] Aided in part by a grant from the American Heart Association.

METHODS

Human subjects without cardiac or respiratory disease came to the laboratory fasting and rested for an hour or more in bed after an indwelling needle was placed in one brachial artery with local anesthesia. Exercise was carried out by two methods for the purpose of producing O_2-debts of various magnitudes. The severe exercise consisted of walking on a motor-driven treadmill; the mild exercise consisted of straight leg raising in the supine position. No further attempt was made to regulate the work done. Exercise lasted from 7 to 15 minutes.

Blood was collected from the brachial artery as previously described (12) at frequent intervals during rest, exercise and recovery. Recovery was followed for one and one-half to two hours. Samples were analyzed for lactate, pyruvate and oxygen as previously described (4). Blood oxygen saturations exceeded 96 per cent in every instance.

Expired air was collected continuously during the severe exertion and intermittently during the mild exercise; collection was continuous from the last minute of exercise throughout the recovery period in all experiments. Timed air collections were carried out through a rubber mouthpiece, low resistance valve and short length of tubing leading to two Douglas bags used alternately. The volume of gas in each bag was determined either by drawing the contents through a carefully calibrated wet-test gas meter at constant pressure and rate of flow, or by determining the dilution volume of a measured quantity of acetylene in the bag. The expired air was analyzed for oxygen and carbon dioxide either in the Haldane apparatus or in the paramagnetic oxygen analyzer (Beckman, Model E2 null-point analyzer with total range 12 to 22 per cent) before and after passage through soda-lime and sodium hydroxide. The analyzer was used at constant pressure and rate of flow. Collection periods were two to five minutes in length. From the volume and composition of inspired and expired air, rate of oxygen consumption was obtained for each period. Total body water was determined in each subject as the dilution volume of 4-aminoantipyrine (13).

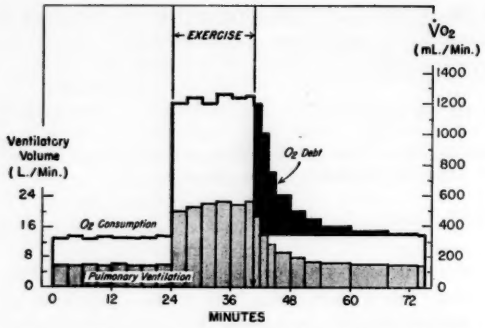

FIG. 1. TYPICAL CHANGES IN VENTILATORY VOLUME AND MINUTE UPTAKE OF OXYGEN BY THE LUNGS IN A SUBJECT DURING STANDING TREADMILL EXERCISE

Total O_2-debt (the shaded area) is calculated as the integral of the $\dot{V}_{O_2}$ curve between the moment exercise ceases and that at which the slope of the curve becomes zero. The measurement of the late portions of this area may be seen to be subject to considerable error since the rates of oxygen consumption are only slightly increased over the plateau rate.

The experimental dogs weighed 10 to 20 Kg. and were anesthetized with 20 mg. per Kg. of chloralose. Exercise was effected by rhythmic electrical stimulation through electrodes inserted into the tissues near the spine and tips of the feet. Collection and analysis of blood and gas samples were the same as for the human subjects except that each recorded blood concentration is the mean of determinations on blood collected simultaneously from the aorta through a femoral cannula and from the right ventricle through a large bore cannula inserted through the jugular vein.

RESULTS

Figure 1 shows a graph of typical oxygen consumption rates plotted for one human subject during an exercise procedure. The shaded area represents the O_2-debt contracted. The O_2-debt remaining at each time, t_n, during recovery is given by the integral of this curve between t_n and t_o, the latter being the time when oxygen consumption ceases to change. When these values are plotted, an "O_2-debt curve" is obtained;[2] this curve is the only one shown in subsequent figures.

Total body lactate was calculated from the concentration of lactate per liter of blood water, by multiplying by the volume of body water. This conversion involves the assumption that the added lactate during exercise has a virtual volume of dilution approximately equal to total body water, an assumption which will be discussed below. Total body lactate in millimoles is then multiplied by its oxygen-equivalence factor, 0.5 mM oxygen (11.2 ml.) per mM lactate. This permits O_2-debt and body lactate to be expressed in the same units for comparison. Neither multiplication, of course, alters the contour of the time curve.

[2] $O_2\text{-debt}_{t_n} = \int_{t_n}^{t_o} (\dot{V}_{O_2} - \dot{V}_{O_{2t_o}})\, dt.$

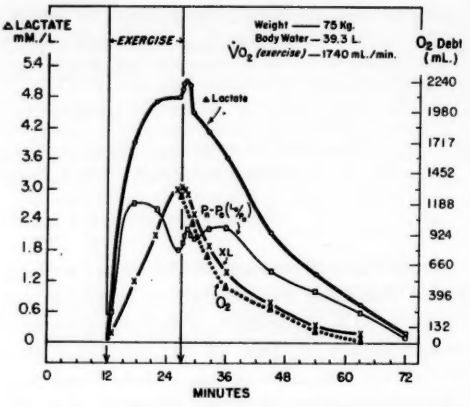

FIG. 2. O_2-DEBT CONTRACTED BY A SUBJECT DURING MODERATELY SEVERE EXERCISE, CALCULATED FROM ΔLACTATE (CHANGE IN BLOOD LACTATE OVER RESTING CONTROL VALUES), FROM EXCESS LACTATE (XL), AND FROM OXYGEN CONSUMPTION MEASUREMENTS (O_2)

Also plotted is the correction factor $P_n - P_o$ (L_o/P_o) (see text). All values are given both in millimolar equivalents of lactate and milliliter equivalents of O_2-debt.

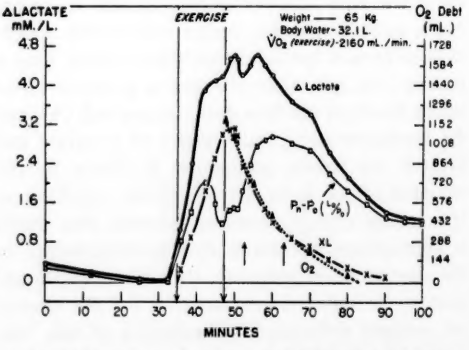

FIG. 3. EFFECT OF CHANGES IN PULMONARY VENTILATION ON THE FORM OF THE O_2-DEBT CURVES FOLLOWING MODERATELY SEVERE EXERCISE IN A HUMAN SUBJECT

The vertical arrows near the baseline indicate the time the subject was instructed to "breathe faster and deeper."

"Excess lactate" or XL is calculated as previously described (4) by the following equation:

$$1) \quad XL = (L_n - L_o) - (P_n - P_o)\ (L_o/P_o)$$

where L_n and P_n are lactate and pyruvate concentrations at any time t_n; L_o and P_o are the levels obtained as a plateau of three or more samples in the near-basal state. The variance of XL, as judged from multiple blood samplings in succession from patients in a near basal state, is ± 0.01 mM per L., or about 5 ml. oxygen, for confidence limits of 95 per cent.

Figures 2 to 5 show plots of $(L_n - L_o)$ (converted to O_2-debt equivalents as described) and also of the second term of equation *1*), $(P_n - P_o)$ (L_o/P_o), similarly converted to oxygen-equivalents. The shape of the latter curve depicts the relative changes in pyruvate observed in the experiment, and its height indicates the quantity of lactate present solely as a result of alteration in pyruvate. The remainder of lactate in excess of this, the presence of which is ascribable to the only other factor affecting lactate production, oxygen lack, is plotted as XL (converted to oxygen-equivalents as described for total lactate).

It can be seen from the figures that total lactate varies with time in a manner wholly different from O_2-debt. Similar observations have been made by Jervell (2). The oxygen equivalents of lactate

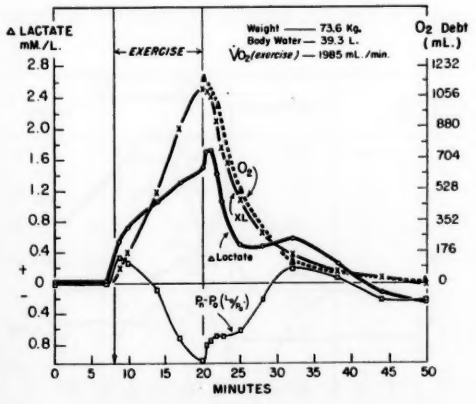

FIG. 4. THE EFFECT ON O_2-DEBT CURVES OF A FALL IN BLOOD PYRUVATE

This phenomenon was occasionally observed during exercise and occurred in athletic young men in physical training; only when pyruvate fell was the lactate curve lower than the true O_2-debt curve.

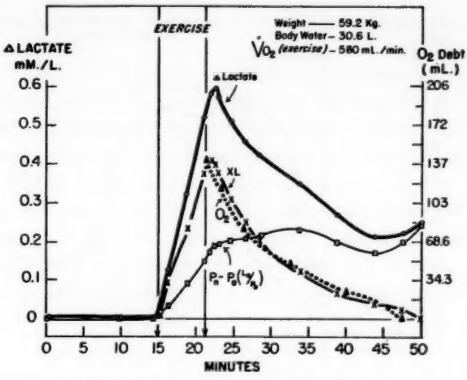

FIG. 5. O_2-DEBT CURVES OF A SUBJECT DOING VERY *MILD* EXERCISE IN THE SUPINE POSITION

Oxygen consumption was increased only 2.5-fold, but the curves after exercise stopped are essentially the same as in severe exercise.

usually far exceeded the O_2-debt, although occasionally they were much less. The absolute values in these figures might be changed by using a different volume of distribution of lactate, but no single value could reconcile the irregular time curves of lactate with those of O_2-debt. Figure 2 shows the smooth falling curve of blood lactate which may be observed in a calm subject, who is thoroughly accustomed to the procedures and whose pulmonary ventilation falls off during recovery in a similar, almost exponential, fashion. This is the type of result seen in trained subjects. Figure 3, however, shows the effect of irregular ventilation during recovery; the lactate curve is no longer smooth. Regularity of lactate curves, when present, is a characteristic probably due to the coincidence of a regularity of ventilation during recovery, and one which we have rarely observed in patients. Pulmonary ventilation with respect to carbon dioxide is known to have a marked effect on lactate production (4).

Figure 4 shows an exercise period in an athletic young man in good physical training, and illustrates a downward error in the lactate estimation of O_2-debt. Such negative lactate errors always occurred in association with decrease of pyruvate, a phenomenon which appears to occur much more commonly in subjects in athletic training, as has been suggested previously (14).

Figure 5 shows the changes in a subject during very mild exercise. Mild exercise has been be-

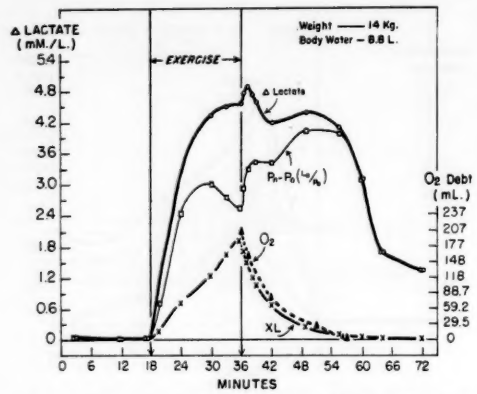

FIG. 6. EFFECT OF ELECTRICALLY INDUCED EXERCISE OF THE ENTIRE BODY IN AN ANESTHETIZED DOG ($\dot{V}_{O_2}$ INCREASED 6-FOLD) SHOWING EXTREME DIVERGENCE OF THE TIME CURVE OF BLOOD LACTATE FROM THAT OF O_2-DEBT

Close correspondence of XL and O_2-debt is seen in spite of the great difference of body weight and proportion of body water from those in the previous figures.

lieved to be fundamentally different from harder exercise (1, 8). The curves, however, appear to be identical with those of severe exertion except in height on the ordinate, but the respiratory O_2-debt, of course, approaches values which are unmeasurable by available techniques, and this may account for the belief in the older literature that mild exercise either produces no oxygen debt (8) or one with a different decay curve (1). Since no basic difference in anaerobic metabolism of tissues is apparent at any grade of exercise, it now seems quite possible that the changes are the same even during exercise too mild to produce a detectable or measurable respiratory oxygen debt. This is suggested also by the fact that excess lactate production is apparent in all subjects after the first two or three muscle contractions of mild supine exercise (within the first minute), as shown in the illustrations.

In all the figures the O_2-debt curves pursue the regular course long found to be characteristic (1, 9). It is notable that the curves of excess lactate are quite similar to those of O_2-debt both in height at any one moment and in contour, and are often nearly superimposable. This is true not only in Figure 2, when the lactate curve is smooth, but also in experiments like that of Figure 3 when total lactate is variable. XL approximates O_2-debt whether total lactate is too high (Figures 2, 3, and 5) or too low (Figure 4). Thus, whether lactate will be found to be excessively high or too low, appears to depend a great deal on how much, and in what manner, pyruvate changes [*i.e.*, curve $(P_n - P_o)\ (L_o/P_o)$].

These same phenomena are illustrated in Figure 6 showing the effects of electrically induced muscular exercise in an anesthetized dog. These experiments extend the observations to include recovery from exercise under complete anesthesia, free of psychic influences and slight muscle movements unavoidable in patients, and extend the range of body size studied down to 10 Kg. weight. Figure 7 illustrates the effect of altering the pump-controlled respiration ($\dot{V}$) of a dog during an exercise period and more clearly emphasizes the complete independence of lactate from O_2-debt. Frequently, arterial blood lactate in unanesthetized human subjects begins to rise prior to the actual beginning of muscle movements, in anticipation of the order to exercise. This phenomenon was not seen in anesthetized dogs.

The sharp peak in lactate curves shown in the figures at the onset of rest was present in all experiments. The highest value for blood lactate was always reached within 90 seconds (average 50 seconds) after the cessation of exercise. There was commonly a similar rise in pyruvate concentration so that XL actually was maximal at the in-

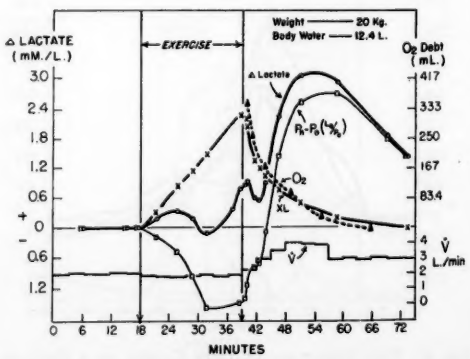

FIG. 7. EFFECTS OF ARBITRARILY ALTERING THE PUMP-CONTROLLED RESPIRATION ($\dot{V}$) OF AN ANESTHETIZED DOG DURING ELECTRICALLY INDUCED MUSCULAR EXERTION, SHOWING THE COMPLETE INDEPENDENCE OF BLOOD LACTATE AND O_2-DEBT

stant of cessation of exercise and fell during the first minute. The maximum rise in pyruvate was much later and varied in time between 7 and 22 minutes (average 12 minutes) after the beginning of rest; (Figure 4 shows an unusual late peak occurring at 35 minutes). This timing of the pyruvate peak is the same as that seen in hyperventilation (8); it is not an inherent characteristic of the rate of uptake of pyruvate by body tissues, however, since the level drops sharply after infusions of pyruvic acid. These same characteristics of the time curve are likewise seen in respiratory hypoxia (15).

In all, 12 "severe" exercise experiments were carried out on five male subjects and one female. Table I shows the body weights, body water contents, rates of oxygen consumption at the end of exercise, and total respiratory O_2-debts. Similar figures are given for the 11 instances of supine (mild) exercise in nine subjects. The errors of estimating O_2-debt from lactate and XL are not adequately represented in the first blood sample

TABLE I

Total O_2-debts calculated from total blood lactate change (ΔL), "excess lactate" (XL), and analysis of respiratory gas (column marked "O_2-debt") in human subjects

Subject	Wt. *Kg.*	Body water *L.*	$\dot{V}_{O_2}$* Exer. *ml./min.*	$\dot{V}_{O_2}$* %Δ	Time *min.*	O_2-debt From $\dot{V}_{O_2}$ *ml.*	O_2-debt From ΔL *mM/L.*	O_2-debt From ΔL O_2 equiv. *ml.*	O_2-debt From XL *mM/L.*	O_2-debt From XL O_2 equiv. *ml.*
Upright exercise (strenuous)										
Ia	75	39.3	2,960	842	20	3,170	4.750	2,090	7.040	3,098
b			1,740	454	15	1,280	5.091	2,240	3.005	1,320
c			995	217	10	410	1.902	837	0.920	405
IIa	82.8	42.4	3,577	971	20	4,550	11.880	5,640	9.538	4,525
b			2,008	498	20	2,300	9.168	4,350	4.613	2,190
c			1,240	271	15	978	4.369	2,072	2.043	970
IIIa	86.8	42.5	1,990	501	15	1,420	6.810	3,240	3.159	1,504
b			2,457	642	15	2,260	3.109	1,480	4.938	2,150
IVa	62.3	29.6	2,985	1,230	15	1,650	5.249	1,740	4.855	1,610
			2,290	842	15	1,830	6.681	2,215	5.601	1,858
V	65.0	32.1	2,160	586	12	1,160	4.619	1,660	3.170	1,140
VI	73.6	39.3	1,985	266	14	1,140	1.702	749	2.491	1,096
Supine exercise (mild)										
I	72.7	36.5	888	205	10	298	1.542	630	0.743	304
II	56.8	27.6	774	250	12	464	0.964	298	1.392	430
III	81.8	43.0	1,007	198	7	222	0.914	440	0.509	245
IV	64.6	34.4	587	110	9	144†	0.649	250	0.364	140
V	77.8	40.3	584	80.2	10	104†	0.554	250	0.217	98
VI	54.5	27.0	880	300	11	385	1.349	408	1.250	378
VIIa	74.6	37.8	489	60.8	9	82†	0.595	252	0.201	85
b			1,205	297	12	508	0.718	304	1.226	519
VIIIa	91.8	43.6	980	187	10	346	0.776	379	0.696	340
b			582	70.2	11	159†	0.578	282	0.297	145
IX	59.2	30.6	580	152	8	140	0.612	210	0.404	138

* $\dot{V}_{O_2}$ = Rate of oxygen consumption.
† See footnote 3.

after exercise, but often showed greater or lesser divergence at other moments of recovery. For this reason a statistical comparison was made between the values by all three methods (respiratory, blood total lactate and blood XL) at each moment when all three were available. In this sense, 150 O_2-debts were estimated by three methods. The "errors" [3] of total lactate as an estimate of O_2-debt averaged 982.5 ml. oxygen ($\sigma = 743.5$) or 119.5 per cent ($\sigma = 90.4$ per cent). The errors of XL averaged -41.3 ml. oxygen ($\sigma = 89.6$) or -5.0 per cent ($\sigma = 10.9$ per cent). The difference between the two means is highly significant, ($p < 0.001$).

Eighty-seven O_2-debts were estimated by three methods in six dogs. The errors of total lactate as an estimate of respiratory O_2-debt averaged 308 ml. oxygen ($\sigma = 188.9$) or 153.2 per cent ($\sigma = 94.0$ per cent). The errors of XL averaged -12.5 ml. oxygen ($\sigma = 16.9$) or -6.2 per cent ($\sigma = 8.4$ per cent). Again the difference was highly significant. The errors in estimating O_2-debt by either method in human subjects and anesthetized dogs were both positive and negative, but a tendency to large positive errors was common for total lactate, while a mean tendency to slight underestimation is evident for XL. This small "negative error" for XL may possibly represent the extent of "positive error" in respiratory O_2-debt measurement due to resaturation of the oxygen pool of the body, a problem which is discussed more fully in the following paper (13), but one which probably is also a feature of exercise experiments (16) to a lesser extent.

DISCUSSION

From previous studies it is clear that lactate production is not controlled by a single factor but by the two factors on the right of equation *2*) in the first paper of this series (4). Lactate changes could be expected to reflect alterations in the oxygen-supply factor only if pyruvate remained constant. If pyruvate changes at the same time as $[DPNH_2]/[DPN]$, the total lactate change will not accurately measure either factor alone. In other words, the system may be subjected to a change in both pyruvate and lactate concentrations simultaneously without any alteration in its balance; this occurs during alkalosis, the infusion of glucose or pyruvate and the administration of insulin or epinephrine (4). On the other hand, the system may also be found to shift its balance to the right, as it does in hypoxia. In actual experience, both changes occur in exercise. The present data give no indication of the basic reason for the alterations in body pyruvate; the control of pyruvate levels in the intact body is too complex to warrant speculation as to their immediate cause at this time. The control of lactate concentrations, however, is far simpler, since lactate takes part in no other equilibrium than that of the lactic dehydrogenase system.

The results obtained in the present experiments depend to some extent upon the methods of blood collection and analysis. If venous blood is sampled from an arm vein during exercise of the legs the concentrations of lactate, pyruvate and XL are significantly lower than those in the mixed venous blood which drains the entire body. The changes shown in the illustrations occur in arterial and mixed venous blood almost immediately on the first movement of exercise (within 30 to 60 seconds) and they continue throughout any period of exertion of this duration. We have found that little or no change occurs in blood draining the resting muscles of the arm, however, for some minutes, if at all. This effect of resting tissues on blood levels may well explain the failure of earlier workers (2, 8, 17–19) who sampled only venous blood, to find any change in blood lactate except in severe exercise. It seems likely that this is also the explanation for some of the anomalously low blood lactate values reported during exercise (5), although the low values which appear to be characteristic of athletes also are an important part of previous studies (8, 19).

Differences in concentration in various parts of the body make the idea of a gross anatomical volume of perfectly uniform distribution of lactate and

[3] The differences from the respiratory O_2-debt values are here referred to as errors, but these differences also include the "error" or variability of the respiratory measurement itself. This technical error may be quite large for small values of O_2-debt. When comparisons are omitted from the statistical calculations for all periods when the elevation of oxygen consumption over basal was less than 10 per cent, however, the mean figures are not significantly different from those given. The small O_2-debts in Table I marked with a dagger were determined with 20 second to 1 minute sampling of expired air in Douglas bags in which both the dead space volume of pure nitrogen and the final volumes were determined by dilution of measured quantities of acetylene, and analyses were all done in the Haldane apparatus.

pyruvate inaccurate, of course, since the distribution is not actually uniform. The present data, however, suggest that the factor relating the concentration of XL in blood water to total body XL is, in fact, approximately equal to the body water content for a great variety of body sizes and grades of exercise. The concept of a virtual volume of distribution is, therefore, useful here.

On the other hand, aside from the mere usefulness of the concept, blood lactate changes have been shown actually to reflect those of tissues accurately (20), and the apparent volume of distribution of lactate in living muscle has been found to be that of the total tissue water content (21). There is evidence that lactate and pyruvate cross certain cell membranes passively (12), and in intact man and dogs, rapidly enough (4) to permit blood determinations by the present methods to depict intracellular events with sufficient reliability if changes are no more sudden than these. However, the application of such a principle to the study of sudden, brief bursts of maximal exercise might be unwarranted.

The present data for recovery periods after exercise indicate that an O_2-debt may be paid off completely while body lactate is still elevated above basal levels or even rising further, *i.e.*, that all the accumulated lactate need not be oxidized when the O_2-debt has disappeared, but only that it bear a certain relationship to body pyruvate so that metabolic oxidations have returned to normal rates. Whether pyruvate is increased or decreased has no apparent effect on O_2-debt. Thus, oxidation of lactate to pyruvate is the step responsible for the consumption of oxygen, and the subsequent fate of the pyruvate seems not to be involved. This is easy to understand in terms of the total rate of flux of the pyruvate pool, which is very large by comparison with the tiny rate of formation from lactate during recovery from exercise (the latter might be equivalent to 15 to 20 ml. of oxygen per minute while the former would be equivalent to 1,000 ml. of oxygen per minute). If this small rate of appearance of pyruvate within cells inhibited pyruvate synthesis from carbohydrate alone, for example, by only 1 to 2 per cent, it would affect neither the pyruvate concentration of the body nor the rate of consumption of oxygen. Thus the O_2-debt equivalent of excess lactate can be assigned a theoretical value of 0.50 mM oxygen per mM XL, the quantity required for conversion to pyruvate. This 11.2 ml. of oxygen per mM XL (1 ml. of oxygen for 8.03 mg. of lactic acid) is remarkably similar to the empirically chosen value of 1 ml. for 7 mg. of lactic acid used by earlier workers (1) for lack of a theoretical value. Use of the latter factor in the present calculations, instead of the theoretical value, would only have increased the already large divergence of lactate from O_2-debt.

While the present data support the idea that lactate production by tissues has no necessary significance with respect to oxygen supply, they suggest that a function of both lactate and pyruvate may, on the contrary, have such significance. The unique information provided by O_2-debt measurement in those very special types of experiments in which the measurement can be made has been appreciated for many years. The phenomenon of O_2-debt formation appears to be a manifestation of a need for oxygen by body tissues during exercise, a need which is not met at the time but satisfied only later during recovery. This unique evidence of oxygen lack appears when blood oxygen content and tension are normal and the rate of delivery of oxygen to the tissues per minute is greater than normal. The metabolic basis of this "energy-borrowing" has, however, remained obscure in spite of attempts to relate it to lactate accumulation. The calculations presented here help to explain the relationship between O_2-debt and tissue anaerobic metabolism; they suggest that the relationship is only slightly more complex than proposed by Hill, Long, and Lupton (1). The error which may occur from using lactate production *per se* as an indication of tissue oxygen lack, however, is very variable and sometimes large. The use of "excess lactate" for this purpose should reduce the error significantly.

It has been suggested that the ratio of lactate to pyruvate actually tends to remain constant at various levels of exertion differing greatly in severity (22, 23), a suggestion which is obviously denied by the present data. This suggestion, however, was derived from extremely variable figures from the point of view of the present computations ($\pm$ 1.0 mM per L. lactate, $\pm$ 0.07 mM per L. pyruvate, and lactate-pyruvate ratios varying by as much as 20). The variability in these reports exceeds the total changes occurring in many of the present experiments. Such variations in the simple ratio, of course, might have negligible

quantitative significance as was assumed in the previous work, or very great significance in any individual case, as pointed out in the first paper of this series (4). Calculated excess lactate, for instance, would be much greater for any given change of lactate-pyruvate ratio in the high range of P_n than at low pyruvate levels. In any event, the present experiments deal with the meaning to be assigned to alterations well within the magnitude regarded as random variance in the studies cited.

At the onset of exercise the rate of oxygen consumption rises to a new level where it remains as long as the rate of exertion is constant. The new V_{O_2}, however, is not achieved instantly but only after one to three minutes of exercise, and the energy expended in performing the exercise during this brief period is sometimes referred to as the "oxygen deficit." In severe or prolonged exercise the "oxygen deficit" is quite small relative to the O_2-debt, but it is more important in lesser exercise. Since the curves of XL accumulation do not indicate that the "oxygen deficit" is supplied entirely by the mechanism which has been the subject of these investigations, it appears that some other source of energy is used if the effort can be assumed to be constant from the first moment of exercise. Actually, the effort may well be less in this first minute than subsequently, but there appears to be no way of determining this with certainty. There is a net diminution of oxygen stored in hemoglobin in the body, and this oxygen presumably supplies a portion of the early energy requirement. In the mild exercise studied the mean change in mixed venous blood oxygen content was 30 ml. per L. in the first two minutes, thus providing about 100 ml. of oxygen during this period in the human subjects. In addition, the slope of the XL curve is greater in the first minute of exercise than subsequently, although this difference is not readily apparent in the illustrations. Finally, evidence from other types of experiment suggest that a net reduction of high energy phosphate compounds in tissues may supply another portion of the oxygen deficit (24), although in the present experiments this has appeared to be small in the mild exercise, after the measurable factors above have been taken into account. The oxygen supplied from hemoglobin and myoglobin is restored during the first minute of resting recovery from exercise and constitutes an "error" in the measurement of O_2-debt as a metabolic phenomenon. The remaining or corrected O_2-debt is found to be slightly more than the quantity of energy made available from the LDH system and this difference may represent the extent to which initial diminutions of the myoglobin oxygen and high energy phosphate pools have failed to be restored before the onset of rest at the end of exercise.

SUMMARY AND CONCLUSIONS

1. Changes in blood lactate in exercise are not correlated with excess oxygen consumption of recovery (O_2-debt) in human subjects or anesthetized dogs, and may err in either direction.

2. Calculated "excess lactate," a function of changes in both pyruvate and lactate, is well correlated with O_2-debt, regardless of the direction or magnitude of the lactate error, erring by an average of -5 per cent ± 11 per cent.

3. The metabolic alterations which are responsible for oxygen debt formation in exercise are essentially similar at all grades of exertion, great and small, and are probably also the same in exertion too slight to produce an oxygen debt which is detectable by available respiratory methods.

REFERENCES

1. Hill, A. V., Long, C. N. H., and Lupton, H. Muscular exercise, lactic acid and the supply and utilization of oxygen. V. The recovery process after exercise in man. Proc. Roy. Soc. B 1924, **97**, 96.
2. Jervell, O. Investigation of the concentration of lactic acid in blood and urine under physiologic and pathologic conditions. Acta med. scand. 1928, Suppl. **24**, 1.
3. Meakins, J., and Long, C. N. H. Oxygen consumption, oxygen debt and lactic acid in circulatory failure. J. clin. Invest. 1927, **4**, 273.
4. Huckabee, W. E. Relationships of pyruvate and lactate during anaerobic metabolism. I. Effects of infusion of pyruvate or glucose and of hyperventilation. J. clin. Invest. 1958, **37**, 244.
5. Margaria, R., Edwards, H. T., and Dill, D. B. The possible mechanisms of contracting and paying the oxygen debt and the role of lactic acid in muscular contraction. Amer. J. Physiol. 1933, **106**, 689.
6. Meyerhof, O., and Meier, R. Über den Milchsäurestoffwechsel im lebenden Tier. Arch. f. d. ges. Physiol. 1924, **204**, 448.

7. Alpert, N. R., and Root, W. S. Relationship between excess respiratory metabolism and utilization of intravenously infused sodium racemic lactate and sodium L (−) lactate. Amer. J. Physiol. 1954, **177**, 455.
8. Owles, W. H. Alterations in the lactic acid content of the blood as a result of light exercise, and associated changes in the carbon dioxide-combining power of the blood and in the alveolar carbon dioxide pressure. J. Physiol. 1930, **69**, 214.
9. Griffith, F. R., Jr., Omachi, A., Lockwood, J. E., and Loomis, T. A. The effect of intravenous adrenalin on blood flow, sugar retention, lactate output and respiratory metabolism of peripheral (leg) tissues in the anesthetized cat. Amer. J. Physiol. 1947 **149**, 64.
10. Hill, A. V., and Lupton, H. Muscular exercise, lactic acid, and the supply and utilization of oxygen. Quart. J. Med. 1922–1923, **16**, 135.
11. Long, C. N. H. Muscular exercise, lactic acid and the supply and utilization of oxygen. XIV. The relation in man between the oxygen intake during exercise and the lactic acid content of muscles. Proc. Roy. Soc. B 1926, **99**, 167.
12. Huckabee, W. E. Control of concentration gradients of pyruvate and lactate across cell membranes in blood. J. appl. Physiol. 1956, **9**, 163.
13. Huckabee, W. E. Use of 4-aminoantipyrine for determining volume of body water available for solute dilution. J. appl. Physiol. 1956, **9**, 157.
14. Yanof, Z. A. Effect of exercise on blood pyruvic acid. Arch. intern. Med. 1943, **72**, 239.
15. Huckabee, W. E. Relationships of pyruvate and lactate during anaerobic metabolism. III. Effect of breathing low-oxygen gases. J. clin. Invest. 1958, **37**, 264.
16. Millikan, G. A. Experiments on muscle haemoglobin *in vivo;* the instantaneous measurement of muscle metabolism. Proc. Roy. Soc. B 1937, **123**, 218.
17. Bock, A. V., Dill, D. B., and Edwards, H. T. Lactic acid in the blood of resting man. J. clin. Invest. 1932, **11**, 775.
18. Friedemann, T. E., Haugen, G. E., and Kmieciak, T. C. Pyruvic acid. III. The level of pyruvic and lactic acids, and the lactic-pyruvic ratio, in the blood of human subjects. The effect of food, light muscular activity and anoxia at high altitude. J. biol. Chem. 1945, **157**, 673.
19. Cook, L. C., and Hurst, R. H. Blood lactic acid in man during rest. J. Physiol. 1933, **79**, 443.
20. Newman, E. V. Distribution of lactic acid between blood and muscle of rats. Amer. J. Physiol. 1938, **122**, 359.
21. Devadatta, S. C. The diffusion of lactate into and from muscle. J. Physiol. 1933, **79**, 194.
22. Stotz, E., and Bessey, O. A. Blood lactate-pyruvate relation and its use in experimental thiamine deficiency in pigeons. J. biol. Chem. 1942, **143**, 625.
23. Goldsmith, G. A. The blood lactate-pyruvate relationship in various physiologic and pathologic states. Amer. J. med. Sci. 1948, **215**, 182.
24. Flock, E. V., Ingle, D. J., and Bollman, J. L. Formation of lactic acid, an initial process in working muscle. J. biol. Chem. 1939, **129**, 99.

RELATIONSHIPS OF PYRUVATE AND LACTATE DURING ANAEROBIC METABOLISM. III. EFFECT OF BREATHING LOW-OXYGEN GASES [1]

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When the oxygen supply to tissues is impaired progressivly, as in continuous reduction of the oxygen content of alveolar air, oxygen tensions in various other parts of the gas transport system proceed to fall progressively also. At some stage in the course of this unbroken continuum a state of "hypoxia" presumably may be said to exist. It is, in any event, a useful concept to suppose that it does, inasmuch as symptoms and dysfunction of organs may appear in the course of diseases of the oxygen transport systems which might be accounted for by hypoxia.

If hypoxia is induced by all diminutions of alveolar Po_2, then this condition may exist with arterial blood saturations reduced by an unmeasurable amount and arterial oxygen contents which are normal or above normal. It seems unprofitable to apply the term in this way because there is no reason to believe that the body suffers in any sense from deprivation of needed oxygen in this situation. It is also true that hypoxia is not necessarily present even at the stage of this continuum in which arterial oxygen content has become significantly decreased (*i.e.,* hypoxemia), since an increase in blood flow may act to prevent any real diminution in Po_2 in the tissues or venous blood. Finally, even if the term hypoxia be reserved for situations in which tissue Po_2 falls, the implication that the tissues are deprived of oxygen under these circumstances is not consistent with the fact that the rate of oxygen consumption may remain unchanged, *i.e.,* the amount of oxygen delivered to and taken up by the tissues per minute is normal.

In the previous papers of this series (1, 2) it has been proposed that the occurrence of anaerobic metabolism in tissues indicates that the rate of supply of oxygen to the interior of tissue cells has fallen below the rate of energy utilization, *i.e.,* has fallen below the rate of oxygen requirement. It seemed possible that if this discrepancy between rates of supply and demand in tissues were taken as the proper indication of physiologic hypoxia, a distinct starting point of hypoxia could be identified in the course of progressive pulmonary or circulatory impairment. In the present study, experimental subjects and animals were given various low oxygen gas mixtures to breathe while anaerobic metabolism, as previously defined (1), was estimated at various blood oxygen tensions.

METHODS

The human subjects were fasting and lay quietly for one to two hour control periods after insertion of a brachial arterial needle. During control determinations of oxygen consumption with air breathing, and during 15 to 30 minute periods breathing prepared gas mixtures, the subjects respired through a rubber mouthpiece and low resistance valve, inhaling from a spirometer or tank demand valve and exhaling into a Douglas bag. All gas mixtures were prepared in pressure tanks and analyzed to determine their exact composition. Oxygen consumption (pulmonary oxygen absorption, $\dot{V}_{O_2}$), pulmonary ventilation, and arterial blood pH, lactate, pyruvate and oxygen determinations were carried out as previously described (2). Experimental animals were dogs lightly anesthetized with chloralose; the same determinations were carried out as in the human subjects, and, in addition, O_2-debts were calculated from the curves of continuously measured oxygen consumption.

Gas mixtures employed contained only oxygen and nitrogen. Oxygen contents were 15.05 per cent (15 per cent O_2), 12.91 per cent (13 per cent O_2), 10.09 per cent (10 per cent O_2), and, in dogs, also 7.12 per cent (7 per cent O_2), or 7.89 per cent (8 per cent O_2), and 5.18 per cent (5 per cent O_2).

The quantity of anaerobic metabolism was determined as "excess lactate" (XL) accumulation, calculated as follows (1):

$$XL = (L_n - L_o) - (P_n - P_o)\ (L_o/P_o)$$

L_o and P_o are the control arterial blood lactate and pyruvate concentrations in mM per L. of blood water, during air breathing; L_n and P_n are the concentrations at

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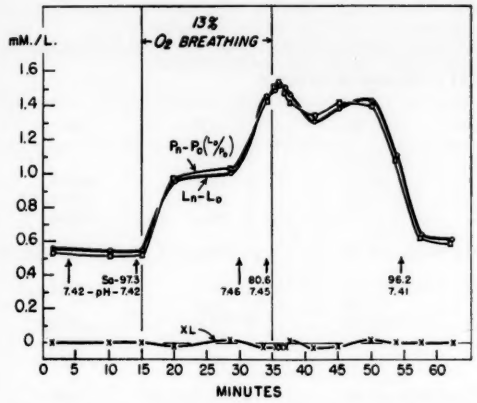

FIG. 1. CHANGES IN ARTERIAL BLOOD LACTATE ($L_n - L_o$) AND PYRUVATE [$(P_n - P_o)(L_o/P_o)$] CONCENTRATIONS, pH AND OXYGEN SATURATION (Sa) IN A NORMAL HUMAN SUBJECT (38 YEAR OLD, MALE, 70.4 KG.) DURING THE BREATHING OF 13 PER CENT OXYGEN IN NITROGEN

Calculated "excess lactate" (XL) remained essentially zero.

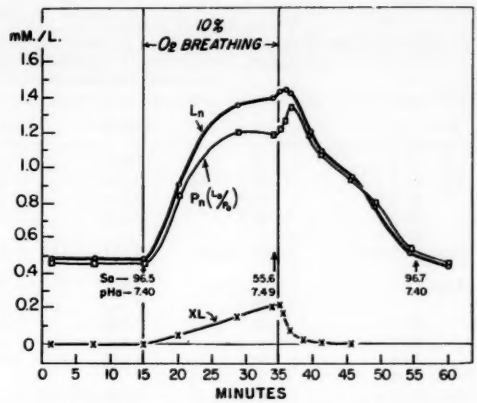

FIG. 2. TYPICAL CHANGES IN ARTERIAL BLOOD LACTATE, PYRUVATE, pH AND OXYGEN SATURATION IN A HUMAN SUBJECT (42 YEAR OLD, MALE, 56.2 KG.) DURING THE BREATHING OF 10 PER CENT OXYGEN IN NITROGEN

The rise in "excess lactate" (XL) distinguishes these results from those obtained with 15 per cent and 13 per cent oxygen.

any other time t_n, *i.e.*, during the breathing of a low oxygen gas mixture.

RESULTS

Figures 1 and 2 illustrate typical experiments in a human subject breathing 13 per cent O_2 and 10 per cent O_2, respectively, for 20 minute periods. In both experiments arterial blood oxygen saturation (Sa) fell, pH rose and blood lactate ($L_n - L_o$) and pyruvate [$(P_n - P_o)$ (L_o/P_o)] concentrations rose. During the breathing of 13 per cent O_2, however, the lactate accumulation was accounted for totally by the rise in pyruvate which occurred during the "hypoxia" since no "excess lactate" (XL) was produced. As shown in Table I this finding was noted in all subjects breathing 15 per cent O_2 and 13 per cent O_2 which, in fact, are very slight reductions of F_{IO_2} (inspired oxygen concentration). Figure 2, however, with more severe reduction of F_{IO_2}, illustrates (curve XL) an increasing discrepancy or excess of lactate production over that to be expected from the observed pyruvate change; this excess lactate accumulation or anaerobic metabolism of tissues during 10 per cent O_2 breathing occurred in 10 of 13 experiments as shown in Table I. Arterial oxygen saturations, Sa, vary in all three groups, presumably from the different ventilatory responses to low oxygen. One of the three subjects who failed to show anaerobiosis during 10 per cent O_2 breathing had a relatively small depression of blood oxygen which might account for the lack of anaerobiosis; the other two, however, did not appear to differ significantly from the remainder of the group as regards arterial oxygen. Two of this group, Nos. 6 and 8, were reexamined on another occasion with the same result. Similarly, Subjects 1, 4, 11, and 13 were restudied once or twice and invariably exhibited XL accumulation with 10 per cent O_2.

The study of graded respiratory hypoxia could be carried much further in animals, as shown in Table II. Dogs showed the same "pseudo-hypoxia" as human subjects when breathing 15 per cent O_2 and 13 per cent O_2, in that blood oxygen values fell and lactic acidemia appeared. Again, however, no excess lactate was present. On the other hand, the breathing of 7 or 8 per cent O_2 and 5 per cent O_2 uniformly resulted in excess lactate accumulation.

The transition appeared to occur at an inspired oxygen content of 10 per cent; part of the experiments in this group showed XL production and part did not. As in the human subjects, measure-

TABLE I

Effects in human subjects of breathing gas mixtures of various oxygen concentrations (FI_{O_2}) on arterial blood oxygen saturation, blood lactate, pyruvate and calculated "excess lactate" (XL) concentrations, pulmonary minute ventilation ($\Delta\dot{V}$), and arterial blood pH

FI_{O_2}		Sa	ΔLactate	ΔPyruvate	XL	($\Delta\dot{V}$)	pH
%		%	*mM/L.*	*mM/L.*	*mM/L.*	%	
15%	1	86.5	0.265	0.053	0	16.7	+0.03
	2	88.9	0.640	0.162	0	20.1	+0.04
	3	89.2	0.449	0.082	0	50.1	+0.06
Mean±range/2		88.2±1.4	0.451±0.188	0.099±0.040	0	29.0±16.7	0.04±0.02
13%	1	83.1	0.340	0.080	0	45.0	+0.05
	2	78.4	0.242	0.063	0	32.4	
	3	86.7	0.560	0.108	0	25.6	+0.04
	4	82.0	0.423	0.085	0	22.1	+0.06
	5	80.6	0.964	0.179	0	40.9	+0.03
Mean±σ		82.2±3.2	0.506±0.283	0.103±0.090	0	33.2±9.76	0.04
10%	1	63.5	0.542	0.118	0.077	67.6	+0.08
	2	69.6	2.650	0.405	0.142	56.2	+0.05
	3	66.4	1.165	0.220	0	30.6	+0.10
	4	67.0	1.264	0.209	0.073	92.9	+0.11
	5	55.6	0.964	0.172	0.204	28.6	+0.09
	6	80.1	1.433	0.293	0	105.0	
	7	50.1	2.681	0.375	0.109	30.2	+0.08
	8	70.7	0.967	0.236	0	56.2	+0.10
	9	70.4	0.984	0.248	0.064	65.9	
	10	72.6	0.699	0.129	0.089	23.4	+0.05
	11	65.4	1.070	0.215	0.050	64.5	+0.07
	12	71.0	1.223	0.223	0.177	70.8	+0.07
	13	65.4	1.571	0.249	0.200	65.0	+0.06
Mean±σ		66.7±7.6	1.324±0.656	0.238±0.083	0.091±0.072*	58.2±24.9	0.08±0.02

* Physiologically this group of figures probably must be regarded as inhomogeneous, containing at least two, and probably n unrelated populations, so that it is not actually susceptible to description by a mean±σ.

ments of molecular oxygen did not show any distinguishing features between the two.

Animals under light chloralose anesthesia have usually exhibited about the same ventilatory response to low oxygen gases as unanesthetized animals, although such figures are very variable for any group. Pulmonary ventilation per minute increased by an average of 22 per cent for FI_{O_2} of 15 per cent and 13 per cent O_2, 38 per cent for 10 per cent O_2, and 76 per cent for 8 and 7 per cent O_2. Average control ventilation was 2,050 ml. per minute per 10 Kg., and recovered to 1,930 ml. per minute per 10 Kg. on returning to air breathing.

Respiratory O_2-debts were measured in the animals for comparison with XL production as an estimate of the adequacy of oxygen supply rate. Previous comparisons have been made and a close qualitative (1) and quantitative (2) correlation has been found. Figure 3 illustrates the method used for measuring O_2-debt during respiratory hypoxia. The heavy line shows the actual curve of continuously measured oxygen uptake, during an experiment with 10 per cent O_2 breathing and no O_2-debt, and illustrates the desaturation period at the onset of low oxygen breathing (ABC) when an apparent reduction of oxygen utilization occurs until the blood and body fluids reach a new oxygen tension. This dip in the curve is the mirror image of that which occurs on the return to air breathing (DEF). For the calculation of O_2-debt, therefore, the area ABC is subtracted from such an area as DGH, when it is present, and the true O_2-debt is indicated by the upper portion. Figure 4 illustrates results obtained in an anesthetized intact dog with spontaneous respiratory control during the breathing of 8 per cent O_2. The respiratory O_2-debt curve is included in this figure and shows the marked divergence between blood lactate changes and O_2-debt, but a close correspondence between XL and the respiratory measurement.

TABLE II

Effect, in anesthetized dogs, of breathing gases of various oxygen concentrations ($F_{I_{O_2}}$)

$F_{I_{O_2}}$		Oxygen debt* *ml. O_2* From $\dot{V}_{O_2}$	From Lactate	From XL	Sa	ΔpH
15%	1	12	25.0	0	74.6	+0.11
	2	0	182.7	0	82.7	+0.08
	3	8	196.1	6.7	76.1	+0.04
	4	0	109.3	4.2	70.4	+0.06
	5	0	299.4	0	85.6	+0.05
	6	14	82.0	0	78.4	+0.04
Mean±σ		5.7±6.5	149.1±95	1.8±5.2	78.0±5.5	+0.06±0.03
13%	1	0	388.0	0	73.2	+0.03
	2	0	92.6	0	70.1	+0.09
	3	10	169.8	14.6	69.8	+0.05
	4	11	34.6	8.2	79.0	+0.11
	5	5	494.1	0	78.6	+0.10
Mean±σ		5.2±5.3	235.8±219	4.6±4.4	74.1±4.5	+0.07±0.03
10%	1	209	668.4	194.6	64.2	+0.05
	2	186	491.0	159.4	63.4	+0.03
	3	298	740.8	299.9	68.7	+0.06
	4	0	498.4	0	72.5	+0.15
	5	0	299.4	0	79.6	+0.12
	6	99	325.0	58.8	65.4	+0.10
	7	84	347.7	104.2	62.1	+0.11
	8	0	194.6	9.4	69.7	+0.09
	9	110	544.0	112.4	64.6	+0.10
	10	0	262.7	4.1	72.4	+0.08
Mean±σ		98.6†	437.2±180.4	94.3†	68.3±5.4	0.09±0.04
8%	1	255	649.7	240.5	55.6	+0.15
	2	309	674.0	310.6	58.9	+0.12
	3	274	590.3	295.7	57.8	+0.10
	4	426	588.4	410.7	51.4	+0.12
	5	330	620.6	355.0	52.9	+0.13
7%	6	198	349.0	209.4	48.9	+0.09
	7	569	785.0	558.9	53.4	+0.10
	8	504	626.0	505.0	55.0	+0.11
	9	486	594.3	490.7	50.6	+0.09
	10	394	399.4	392.7	48.4	+0.10
Mean±σ		375.5±121‡	587.7±127.1	375.9±116.8‡	53.3±3.6	0.11±0.02
5%	1	644	704.9	632.4	47.4	+0.05
	2	695	799.4	680.7	48.9	+0.03
	3	704	857.6	710.8	46.2	+0.09
	4	394	670.4	410.0	47.0	+0.10
Mean±range/2		609.2±155	758.0±93.6	608.4±150.4	47.4±1.4	+0.07±0.04

* Total O_2-debts contracted in the periods studied (30 to 40 minutes) are calculated from the recovery oxygen consumption ratio ($\dot{V}_{O_2}$), from blood lactate changes and from both lactate and pyruvate (XL) for comparison.

† This mean serves only to denote the relative order of magnitude. On physiologic grounds it seems likely that this group is not a single population of figures which can be described by a mean±σ, but is at least two and probably n unrelated populations. The values of σ are 105 and 100.9, respectively, for $\dot{V}_{O_2}$ and XL.

‡ Again, as in (†), this variance does not describe the reliability of the value in one individual, which is about 10 per cent for O_2-debt and less than 5 per cent for XL, but the variability of response between animals.

The same correlation between XL and O_2-debt found in exercise (2) may also be noted in the 35 experiments in Table II. No O_2-debt was contracted during 15 per cent or 13 per cent O_2 breathing in any experiment, or in those animals breathing 10 per cent O_2 in which no XL accumulation was found. In the remainder the respiratory O_2-debt was of the same order of magnitude as the XL accumulation. As in exercise (2), total blood lactate showed no such correlation with

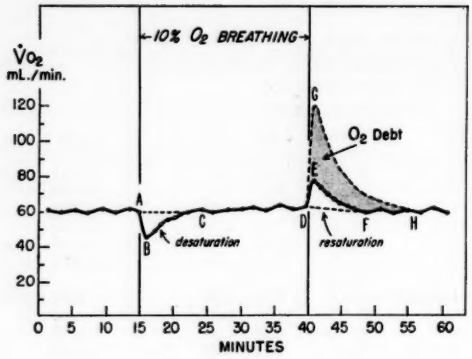

FIG. 3. TYPICAL CHANGES IN CONTINUOUSLY MEASURED OXYGEN CONSUMPTION RATE ($\dot{V}_{O_2}$) IN A DOG BREATHING 10 PER CENT OXYGEN IN NITROGEN WHEN NO MEASURABLE RESPIRATORY O_2-DEBT WAS CONTRACTED (A TO F), SHOWING THE ESSENTIALLY IDENTICAL "DESATURATION" AND "RESATURATION" CURVES

Curve DGH is taken from another experiment in which an O_2-debt was contracted and is superimposed to illustrate the method of deriving O_2-debt.

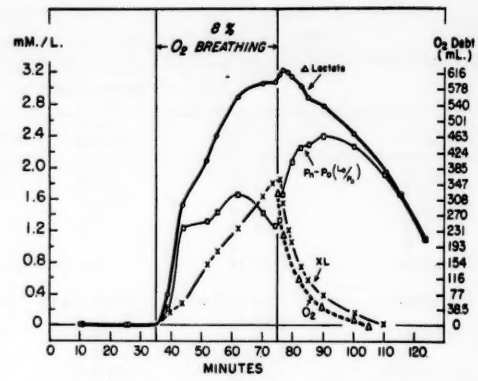

FIG. 4. EFFECT OF BREATHING 8 PER CENT OXYGEN ON THE RELATIONSHIPS OF LACTATE, XL AND O_2-DEBT IN A DOG

O_2-debt. The mean errors of estimating 210 O_2-debts were: *a*) for total lactate, 276.4 ml. oxygen ($\sigma = 178.2$) or 172.1 per cent ($\sigma = 115.2$ per cent); *b*) for XL, 17.2 ml. oxygen ($\sigma = 17.9$) or 11.4 per cent ($\sigma = 12.1$ per cent). The difference between the two sets of errors is highly significant ($p < 0.001$). The errors of lactate were all positive; those of XL were both positive and negative (algebraic mean = +3.2 ml.). The lactate error showed a rough tendency to follow the pH effects of the different gases, averaging 270.5 ml. for the groups breathing 13 to 15 per cent O_2, 322.9 ml. for 10 per cent O_2, 284.3 ml. for 7 to 8 per cent O_2, and 178.9 ml. for 5 per cent O_2.

DISCUSSION

An increase in blood lactate occurs in human subjects as a result of breathing almost any gas mixture with an oxygen content less than that of air. If previous results (1) are considered along with the present ones, it may be inferred that this lactic acidemia occurs whenever hyperventilation with respect to carbon dioxide occurs. As shown in the present data, the mean rise in lactate becomes greater with each further reduction in the oxygen tension of inspired air. Similarly, arterial blood and tissue P_{O_2} change gradually (with considerable variation) as the impediment to oxygenation becomes more severe. From these measurements one cannot identify a transition from adequate oxygenation to hypoxia, if it occurs, and in spite of these changes the rate of oxygen supply continues unaltered. None of the subjects or animals showed decrease of the total rate of metabolic uptake of oxygen from the breathing of low oxygen gases, except terminally in some of the animals breathing 5 per cent O_2. From the $\dot{V}_{O_2}$ alone, it might be concluded that no deficiency of oxygen occurred in any of the experiments. An objection might be raised, however, that this measurement is relatively imprecise, and the $\dot{V}_{O_2}$ itself somewhat variable from moment to moment, so that a reduction of a few ml. per minute for a prolonged period would not be recognizable, especially in view of the small increases in $\dot{V}_{O_2}$ resulting from the increased work of breathing.

Excess lactate production differed from the other observations in this spectrum of changes. At all oxygen concentrations above approximately 10 per cent (equivalent to 18,000 feet altitude) no XL accumulation occurred in human subjects or animals. This encompassed in the present observations arterial oxygen saturations of 88.2 ± 1.4 per cent and 82.2 ± 3.2 per cent for the human subjects and for the animals 78.0 ± 5.5 per cent and 74.1 ± 4.5 per cent. But with all concentrations below 10 per cent O_2, XL accumulation uniformly occurred; values of Sa were 53.3 ± 3.6 per cent and 47.4 ± 1.4 per cent. Inspired gas of

about 10 per cent oxygen content appeared to be the transition zone in both species, with Sa figures of 66.7 ± 7.6 per cent and 68.3 ± 5.4 per cent at this apparently critical level. At this level, the cardiopulmonary responses in some individuals seem to have been able to prevent a fall in oxygen supply below the critical needs of the tissues, while in others they were unable to compensate, and the tissues had to resort to anaerobic metabolism and contraction of O_2-debt. It may be inferred that the "efficiency" of the individual oxygen transport system became critical to the peripheral body tissues at arterial oxygen saturations between 60 and 74 per cent or Po_2 of 26 to 32 mm. of mercury; this inferrence has been confirmed in studies of patients with cardiovascular or pulmonary disease which will be reported separately.

Previous investigations of respiratory hypoxia are difficult to compare with the present study for several technological reasons which have been discussed previously (1): *a*) pyruvate and lactate in arm venous blood may change very little, or fail to change altogether, during stimuli which lead to considerable alterations in concentrations in mixed venous and arterial blood; *b*) delay in collection and denaturation of blood samples may produce *in vitro* errors of such magnitude as to mask small *in vivo* changes; *c*) older methods of analysis appear to give systematically different and more variable results; and *d*) failure to start from absolutely basal levels seriously obscures fairly large changes and may even give changes in an opposite direction.

Jervell (3) found normal lactate values during the breathing of low oxygen gases of more than 7.5 per cent; below this an increase was detected with his methods. Marked increases, however, had been reported (4) at high altitudes.

Resting blood lactates appear to have been about normal at rest at an altitude of 14,000 feet (equivalent to about 12 per cent oxygen) in studies by Dill and co-workers (5), but rose excessively on exercise; pulmonary ventilation also rose excessively, however, and this phenomenon may itself account for relatively large rises of lactate (1).

Myerson, Loman, Edwards, and Dill (6) found a slight and variable increase in arterial blood lactate during the breathing of 9 per cent oxygen for 10 minutes, but the magnitude of increase was unrelated to degree of anoxemia, and oxygen supply was concluded to be unaffected in the experiments. An insignificant rise in venous blood lactate during 9 per cent oxygen breathing has also been found (7). Edwards (8) noted a slight increase during rapid ascent to 9,000 feet (14.6 per cent oxygen), but normal values after reaching 20,000 feet (9 per cent oxygen), when arterial oxygen was much lower. Friedemann, Haugen, and Kmieciak (9), studying one subject, observed some increase in both lactate and pyruvate of venous blood at all altitudes (10,000 to 22,000 feet, 14 to 8 per cent oxygen) while the lactate-pyruvate ratio either showed no significant change, or else rose or fell slightly. Bay and co-workers (10) found no difference between sea level and 10,000 or 15,000 feet (14 per cent and 11 per cent oxygen) in resting blood lactate, pyruvate or the ratio. Both lactate and pyruvate rose more with exercise during respiratory "hypoxia," but the changes in lactate-pyruvate ratio were unrelated to the presence of hypoxia. Other workers (11) have likewise found that lactate-pyruvate ratio bears no relation to hypoxia during exercise, although a systematic increase in absolute lactate response occurs with increasing anoxemia. Presumably this may be attributable to the increasing degree of hyperventilation which occurs with exercise at low oxygen tensions (5, 12). Herber (13) found no increase in arterial blood lactate of asphyxiated dogs until arterial oxygen content had fallen below 5.0 volumes per cent (perhaps 30 per cent saturation); but this hypoxia was associated with marked carbon dioxide retention, by contrast with the alkalosis seen with unimpaired respiration. In another investigation (14) venous blood lactate was slightly elevated and the lactate-pyruvate ratio was apparently not consistently or significantly altered at 19,000 and 23,000 feet (9.5 and 7.8 per cent oxygen) except when a high carbohydrate diet had been eaten, but no control values for the latter are given, although fasting seemed to have a similar effect. These results, taken as a whole, do not provide any generalization regarding adequacy of oxygen supply at any inspired oxygen tension.

The interrelationships of total lactate, XL, and O_2-debt previously reported under various conditions (1, 2) are confirmed for respiratory hypoxia in the present experiments. The conditions de-

scribed here differed significantly from exercise in that they produced no change in tissue oxygen requirements, and led to a primary reduction in blood oxygen content rather than a normal value. Tissue carbon dioxide was reduced rather than increased, and all the organs, rather than primarily the muscles, were affected. The common denominator was a *relative* oxygen lack. As before, total lactate failed to be related to O_2-debt. It seems likely that there is no necessary relationship at all between tissue lactate production *per se* and oxygen deficiency or anaerobic metabolism, whether in exercise, glucose infusion or respiratory hypoxia. When a more complete view of the status of all the components of the lactic dehydrogenase system is taken, however, as has been attempted in the calculation of "excess lactate," a satisfactory qualitative and quantitative reconciliation is achieved between the independent estimate of respiratory O_2-debt and tissue metabolic processes estimated in body fluids.

The body fluids in question here require some comment, inasmuch as the measurements of relative pyruvate and lactate increments are carried out on blood. It should be emphasized that the blood draining all body tissues, mixed in the central circulation, and sampled as it leaves the heart, provides determinations which we regard as representing the conceptual mean of the entire body at a particular moment prior to the sampling. Such a concept does not require that the body be a homogeneous medium. At the same time, of course, such a sample provides no information about events in any functional subdivisions which may be present, but only the net results of all these events. Studies of many individual organs during respiratory hypoxia, for instance, clearly indicate that certain sites may predominate in XL production at a given time and others, notably skeletal muscle, may at the same time be active in removing XL from the blood; and the location of these respective sites may change from moment to moment. This variation in different organs involves new aspects of the subject that will be presented at a later time, but the time-scale of these shifts has not been found to be rapid, being of the order of five or more complete circulation times, so that the concept of the integrated blood drainage of the "mean body" is not impaired in the results presented in this and the preceding papers. The further question of local blood-tissue concentration equilibrium is discussed elsewhere (1, 2).

It will be recognized that estimates of respiratory O_2-debt by the present method probably are somewhat more variable than in exercise (2) because of the necessity of measuring the "desaturation area" of the $\dot{V}o_2$ curve illustrated in Figure 3. This diminution in pulmonary oxygen absorption occurs during the initial fall in blood oxygen and disappears as the blood oxygen content levels off at a new value after about 10 minutes, and is presumably due to the supply of oxygen coming from this desaturation of blood and body fluids since its magnitude is related to $F_{I_{O_2}}$. Reduction of myoglobin, cytochrome oxidase, cytochromes and flavoproteins might also conceivably have taken part in this process if P_{O_2} in some tissue cells fell to extremely low levels, although these substances are unaffected by oxygen tensions of the order of those in the blood (15, 16). The absence of any detectable notch in the XL curve suggests that DPN did not take part in the "desaturation," and presumably the DPN-coupled metabolic systems were unaffected. The question arises, however, whether a reduction in the high energy phosphate pool accompanied the desaturation phase (17).

In that event an actual decrease in metabolic oxygen consumption would have occurred, so that part of the "desaturation area" would be actual O_2-debt and should not have been subtracted from the excess oxygen uptake of recovery. An approximate computation of the change in oxygen pool size can be made, however, from *a*) the known change in arterial and mixed venous blood P_{O_2} and the total circulating hemoglobin mass (assuming 70 per cent of the blood to be venous), plus small additional portions from *b*) change in alveolar P_{O_2}, *c*) the change in plasma P_{O_2}, assuming this change to be uniform in extracellular fluid, and *d*) the desaturation of the intracellular fluid, assumed to have a mean P_{O_2} of 15 to 20 mm. Hg and to undergo a similar percentage change. These calculated changes approximately accounted for the desaturation area; the errors of the approximation were both positive and negative and had an arithmetic mean of 15 per cent. The entire desaturation area is small by comparison with the O_2-debt area in experiments with 5 to 8 per cent oxygen breathing, and 15 per cent of this area represents a negligible variance. The

absence of any large or consistent error in this approximation suggests that if reductions of the creatine phosphate-adenosine triphosphate pool of the whole body occurred they were of insignificant magnitude.

SUMMARY AND CONCLUSIONS

1. Blood total lactate concentration bears no relationship to severity of respiratory hypoxia, but calculated "excess lactate" corresponds closely to the magnitude of the O_2-debt developed in this as in other types of hypoxia.

2. Progressive reduction of oxygen content of inspired air in human subjects and anesthetized animals leads to gradual alteration in blood oxygen, pH, lactate and pyruvate; but excess lactate is absent until $F_{I_{O_2}}$ is reduced to a critical value of about half (arterial blood oxygen saturation reduced to 60 to 74 per cent, P_{O_2} to 26 to 32 mm. Hg) and thereafter is produced in increasing amounts as oxygen supply diminishes.

3. It is suggested that a wide range of hypoxemia, including the range of visible cyanosis in many subjects, is not associated with hypoxia or deficiency of oxygen in body tissues, and that caution should be exercised in ascribing symptoms or signs to this particular aspect of pulmonary or circulatory disease.

REFERENCES

1. Huckabee, W. Relationships of pyruvate and lactate during anaerobic metabolism. I. Effects of infusion of pyruvate or glucose and of hyperventilation. J. clin. Invest. 1958, **37**, 244.
2. Huckabee, W. Relationships of pyruvate and lactate during anaerobic metabolism. II. Exercise and formation of O_2-debt. J. clin. Invest. 1958, **37**, 255.
3. Jervell, O. Investigation of the concentration of lactic acid in blood and urine under physiologic and pathologic conditions. Acta med. scand. 1928, Suppl. **24**, 1.
4. Barcroft, J. The Respiratory Function of the Blood, 2 vols. London, Cambridge Univ. Press, 1925–1928.
5. Dill, D. B., Edwards, H. T., Fölling, A., Oberg, S. A., Pappenheimer, A. M., Jr., and Talbott, J. H. Adaptations of the organism to changes in oxygen pressure. J. Physiol. 1931, **71**, 47.
6. Myerson, A., Loman, J., Edwards, H. T., and Dill, D. B. The composition of blood in the artery, in the internal jugular vein, and in the femoral vein during oxygen want. Amer. J. Physiol. 1931, **98**, 373.
7. Bock, A. V., Dill, D. B., and Edwards, H. T. Lactic acid in the blood of resting man. J. clin. Invest. 1932, **11**, 775.
8. Edwards, H. T. Lactic acid in rest and work at high altitude. Amer. J. Physiol. 1936, **116**, 367.
9. Friedemann, T. E., Haugen, G. E., and Kmieciak, T. C. Pyruvic acid. III. The level of pyruvic and lactic acids, and the lactic-pyruvic ratio in the blood of human subjects. The effect of food, light muscular activity and anoxia at high altitude. J. biol. Chem. 1945, **157**, 673.
10. Bay, E., Barron, E. S. G., Adams, W., Case, T., Halstead, W. C., and Ricketts, H. T. The behavior of blood lactate and pyruvate with exercise at sea-level and at altitude. Part III. National Research Council Report No. 344, Committee on Aviation Medicine, 1944.
11. Tepperman, J., and Tepperman, H. M. On the blood lactic acid response to measured exercise in hypoxic human subjects. J. clin. Invest. 1948, **27**, 176.
12. Douglas, C. G., Haldane, J. S., Henderson, Y., and Schneider, E. C. Physiological observations made on Pike's Peak, Colorado, with special reference to adaptation to low barometric pressures. Philos. Trans. B 1913, **203**, 185.
13. Herber, F. J. Metabolic changes of blood and tissue gases during asphyxia. Amer. J. Physiol. 1948, **152**, 687.
14. Friedemann, T. E., Ivy, A. C., Kinney, V. M., Blumsheft, B., and Harris, S. C. Work at high altitude. VI. The effect of diet and other factors on the rise of lactic and pyruvic acids and the lactate-pyruvate ratio in human subjects at simulated high altitudes. Quart. Bull. Northw. Univ. med. Sch. 1949, **23**, 448.
15. Baumberger, J. P. The relation between the "oxidation-reduction potential" and the oxygen consumption rate of yeast cell suspensions. Cold Spr. Harb. Symp. quant. Biol. 1939, **7**, 195.
16. Millikan, G. A. Muscle hemoglobin. Physiol. Rev. 1939, **19**, 503.
17. Flock, E. V., Ingle, D. J., and Bollman, J. L. Formation of lactic acid, an initial process in working muscle. J. biol. Chem. 1939, **129**, 99.

CHROMATOGRAPHY OF SERUM PROTEINS IN NORMAL AND PATHOLOGIC SERA: THE DISTRIBUTION OF PROTEIN-BOUND CARBOHYDRATE AND CHOLESTEROL, SIDEROPHILIN, THYROXIN-BINDING PROTEIN, B_{12}-BINDING PROTEIN, ALKALINE AND ACID PHOSPHATASES, RADIO-IODINATED ALBUMIN AND MYELOMA PROTEINS

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Chromatographic techniques have recently been introduced which provide new approaches to serum protein separation and characterization (1–4). Sober, Gutter, Wyckoff, and Peterson, using diethylaminoethyl (DEAE) cellulose, described the initial application of anion-exchange cellulose chromatography to the fractionation and identification of serum proteins (1, 2). Their study indicated the great potential usefulness of anion-exchange cellulose chromatography for the study of proteins, but considerable time and work was required to carry out a single determination.

The system of analysis employed in these studies was adapted from a technique developed during a continuing investigation by Drs. Peterson and Sober (5) of chromatographic methods for protein separation. It differs from the technique described previously (2) principally in the use of a single gradient elution system and in modifications introduced in the present study for the purpose of decreasing the initial loss of euglobulins and reducing the time and work involved. These modifications made feasible the chromatographic examination of multiple individual serum samples and serum fractions. Serum samples of 20, 10, 2, and 1 ml. have been examined by this method.

The present study reports the initial findings in the application of the modified chromatographic procedure to the examination of normal human serum and serum obtained in various diseases. The chromatographic fractions of normal serum were characterized by electrophoresis and by carbohydrate and cholesterol content. The chromatographic behavior of siderophilin (iron-binding protein, transferrin), vitamin B_{12}-binding protein, thyroxin-binding protein, alkaline and acid phosphatases, myeloma protein and radioiodinated human serum albumin was also determined.

METHODS

Sample. Early morning fasting blood samples were obtained and allowed to clot at room temperature for about four hours. Serum was removed after centrifugation, and dialysis was started immediately or the sample was frozen for future analysis. The serum to be chromatographed was dialyzed at 4° C. for six or more hours with internal stirring against at least three changes of 15 to 30 volumes each of pH 8 sodium phosphate buffer that was 0.01 M for phosphate. After dialysis, a small amount of white flocculant precipitate, comprising 0.5 to 3 per cent of the total serum protein was separated by centrifugation at 2 to 4° C. The volume of the clear amber supernatant serum was measured and an aliquot was set aside for analytic purposes.

Column preparation. Anion-exchange cellulose adsorbent, DEAE-SF, which had been prepared as a single large batch, was used throughout these studies.[1] After each chromatogram was completed, the adsorbent was regenerated (1), employing the following sequence of washes: 0.3 M monosodium phosphate, 0.5 N sodium hydroxide alternating with water, acidified alcohol to remove nonsaponifiable lipid, and finally alkali and water washes prior to adjustment of the adsorbent to pH 8. The adsorbent was then rinsed with at least 1 L. of 0.01 M pH 8 sodium phosphate buffer. A dilute suspension of the adsorbent was added to a 2.5 cm. O.D. pyrex column and packed with the aid of air pressure (gradually increased to 10 p.s.i. to maintain an effluent flow of 1 ml. per 15 to 30 seconds), then transferred to a cold room (4° C.) and washed further with buffer. Approximately

[1] Solka-Floc SW-40B was sieved to obtain a 230 to 325 mesh fraction that was used as the starting material in preparation of the DEAE-cellulose, which was generously made available by Drs. E. A. Peterson and H. A. Sober. This preparation contained 1.34 per cent nitrogen (0.96 mEq. per Gm.).

TABLE I

Conditions employed for chromatography of a range of serum volumes

Adsorbent (*Gm.*)	Serum sample (*ml.*)	Column size (*cm.*) Adsorbent length	×	O. D. of tube	Round flask*	Erlenmeyer flask†	Flow rate (*ml./hr.*)
16	20	36.5	×	2.5	1,000	500	20–25
9	10	21	×	2.5	500	250	15–20
3	2	29	×	1.4	250	125	10
					110	55	10
2	1	27	×	1.2	110	55	10

* Size of flask and volume (ml.) of 0.01 M pH 8 phosphate buffer.
† Size of flask and volume (ml.) of 0.30 M monosodium phosphate.

16 Gm. of adsorbent was used to prepare columns packed 36 cm. high.

Development of the chromatogram. Elution of proteins was accomplished by a system which produced a continuous rise in phosphate molarity from 0.01 to 0.30 M and a fall in pH from 8.0 to 4.5 in the buffer entering the column. This was achieved by the apparatus (5) illustrated in Figure 1, which consisted of a cone-shaped reservoir (Erlenmeyer flask) filled with 0.3 M monosodium phosphate connected by a syphon to a spherical mixing chamber (round bottom flask) filled with 0.01 M pH 8 sodium phosphate buffer. The syphon and tubing leading to the column were filled with this latter buffer. Dialyzed serum was applied to the column as previously described (2), and continuous buffer flow from the gradient elution apparatus was started. With the 16 Gm. adsorbent columns a flow rate of approximately 20 to 25 ml. per hour was achieved with the flasks 32 inches above the top of the adsorbent column. Effluent fractions of 4 ml. or 8 ml. were collected in an automatic fraction collector [2] from the time of introduction of serum onto the adsorbent.

For smaller serum samples (or equivalent amounts of protein) the equipment was modified as outlined in Table I. The relative ratio of the capacity of the round bottom and the Erlenmeyer flasks was kept at 2:1. In the smaller chromatograms 3 ml. fractions were generally collected.

Examination of the effluent. The effluent fractions were routinely examined for protein content in a Beckman DU spectrophotometer by measuring the optical density at 280 mμ. Biuret determination of the total protein in serum was made by the method of Gornall, Bardawill, and David (6). Dilute solutions were tested by the modification described by Lever and co-workers (7). Each series of biuret readings was standardized against a preparation of crystalline bovine serum albumin which had been checked by a micro-Kjeldahl method. pH measurements were made at room temperature. Ultrafiltration, when required, was carried out at 4° C. (2).

Paper electrophoretic determinations were made on 0.008 ml. of whole serum or 0.01 to 0.05 ml. of concentrated effluent (usually 0.1 to 0.5 mg. protein). Eight 2.9 by 30.0 cm. Whatman 3 MM filter paper strips moistened with 0.075 μ pH 8.6 diethyl barbiturate buffer were run simultaneously in one cell of the hanging strip type (8) at room temperature for 18 hours at a constant current of 6 ma. per cell and under a potential of 68 to 72 volts. The strips were stained with bromphenol blue (9) and evaluated by transmission densitometry with an integrating scanner,[3] using a cam which provided a plot linear with sample concentration. In each electrophoretic cell a normal human serum was utilized as a standard on at least one paper strip. Electrophoretic identification of components on other strips was made relative to the movement of the components in this standard. In order to better detect and quantify components present in small amounts, a crystalline bovine albumin "carpet" (0.01 ml. of a 3 per cent solution) was applied and subjected to

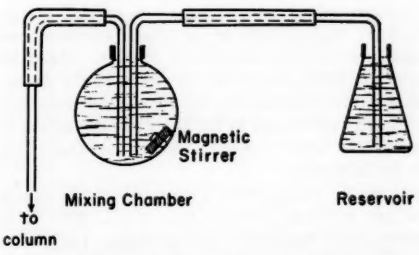

FIG. 1. DIAGRAM OF THE MIXING-CHAMBER AND RESERVOIR UTILIZED DURING CHROMATOGRAPHY

At the beginning of the chromatographic procedure the round bottom flask is filled with 0.01 M pH 8 phosphate buffer and the Erlenmeyer flask with 0.3 M monosodium phosphate in a ratio of 2:1, respectively. The fluid level was the same in each flask. A small notch (not illustrated) is present at the orifice of each glass tube in the mixing chamber and reservoir and serves to prevent sealing and facilitate mixing.

[2] Gilson Medical Electronics, Inc., Madison, Wisconsin.

[3] Analytrol (R), Spinco Division, Beckman Instruments, Inc., Belmont, California.

electrophoresis for five hours prior to application of the fractions to be analyzed.

Localization of specific properties. Serum to be examined was prepared as described above and a standard chromatographic procedure was employed, utilizing about 20 ml. of serum, 16 Gm. of adsorbent, a flow rate of 20 to 25 ml. per hour, effluent collected in 4 ml. fractions, and gradient elution with 1,000 ml. of 0.01 M pH 8 sodium phosphate in the round bottom flask and 500 ml. of 0.30 M monosodium phosphate in the Erlenmeyer flask. This procedure was altered only in the study of thyroxin-binding protein as described below.

Protein-bound carbohydrate distribution was assessed by a modification of the thymol-sulfuric acid method of Shetlar and Masters (10). The results expressed here are considered to represent protein-bound hexose. Serums to be tested for glycoprotein distribution were dialyzed against an extra two changes of buffer to assure the removal of free sugar.

Cholesterol measurements were made according to the method of Abell, Levy, Brodie, and Kendall (11). Aliquots of the effluent fractions were pooled so that the chromatogram was divided equally among 40 fractions. These pools were then lyophilized to reduce the volume prior to cholesterol analysis.

Siderophilin (iron-binding protein, transferrin) was located by means of tracer amounts of radioactive iron. To 25 ml. of fresh normal serum, which contained 73 μg. per cent iron and 300 μg. per cent total iron binding capacity (12), was added 24 μg. (25 μc.) of iron as ferric chloride. Unlabeled ferric chloride was then added to assure saturation of the serum iron-binding capacity. Twenty ml. of the dialyzed serum was added to the adsorbent column and chromatography was carried out in the usual way. Radioactivity in the effluent fractions was determined in a well-type scintillation counter. About 30 per cent of the radioactivity was recovered in the eluate. The remaining radioactive iron, having become separated from the protein and bound to the adsorbent during chromatography, was later removed from the adsorbent and found to be dialyzable.

The vitamin B_{12}-binding protein was identified by means of B_{12} assays utilizing L. leichmanii ATCC 4797 in a modification of the standard U.S.P. procedure (13).

Serum with high acid or alkaline phosphatase activity was obtained from patients with metastatic neoplasms. The whole serum and individual fractions were assayed for alkaline and acid phosphatase activity by adaptations of the method of Bessey, Lowry, and Brock (14). Phosphate was removed from the chromatographic fractions prior to acid phosphatase analysis by thorough dialysis against pH 5, 0.15 M sodium acetate buffer and prior to alkaline phosphatase analysis by dialysis against a 0.05 M sodium acetate: 0.10 M sodium chloride solution. The alkaline and acid phosphatase recoveries in these chromatograms were 125 and 91 per cent, respectively.

Thyroxin-binding protein was located by utilization of radioiodine-labeled thyroxin of at least 90 per cent purity. Normal serum (20 ml.) mixed with 6.2 μg. of labeled thyroxin was allowed to equilibrate overnight in the refrigerator before dialysis. Serum chromatography differed from the usual procedure in the use of a 750 ml. Erlenmeyer reservoir containing 0.3 M monosodium phosphate and a beaker 10 cm. in diameter containing 1 L. of 0.01 M pH 7.5 phosphate buffer as the mixing chamber. Effluent fractions of 6 ml. were collected, and the radioactivity was determined in 4 ml. aliquots by means of a well-type scintillation counter.

The electrophoretic localization of the radioactive thyroxin in the chromatographic fractions was ascertained by standard electrophoretic procedures and by reverse-flow zone electrophoresis (15). After the strips were dried, radioactivity was measured with an end window Geiger-Müller counter, employing a continuously recording counting-rate meter (15), and the distribution of radioactivity was compared with protein distribution as determined by bromphenol blue staining.

The distribution of radioiodine-labeled albumin [4] was determined by addition of 0.1 ml. of solution containing 1.0 mg. (20 μc.) of albumin to 27 ml. of fresh normal serum with a total protein concentration of 6.6 Gm. per cent of which 54.5 per cent was albumin on electrophoretic examination. After dialysis 20 ml. of serum was chromatographed. The radioactivity of the serum and chromatographic fractions was determined in a well-type scintillation counter and results were corrected for decay. The radioactivity recovered following chromatography was 91 per cent of the amount applied to the column.

RESULTS

Normal serum chromatogram

Chromatographic analysis has been performed as described above on 23 individual serums (20 ml. samples) from normal adults and also on 14 serums from patients representing a variety of diseases. A representative normal chromatogram is reproduced in Figure 2. A number of peaks of protein distribution are evident.

Electrophoretic characterization of the chromatogram (Figure 2) indicates that the usual electrophoretic components of serum have been subdivided by the chromatographic procedure. Also, it is apparent that most of the chromatographic regions are composed of several electrophoretically separable components.

Recovery of protein from the chromatogram was essentially complete. Table II compares the recovery of electrophoretic components in the

[4] RISA was obtained from the Abbott Laboratories, North Chicago, Illinois, and is described by the manufacturers as containing 1 iodine: 5½ molecules of albumin, less than 0.5 per cent dialyzable radioactivity, and as being homogeneous by electrophoretic analysis.

TABLE II

Recovery of serum electrophoretic components in the chromatogram effluent

Component		Chromatogram* protein (%)	Electrophoretic† protein (%)
Faster-than-albumin		0.17	
Albumin		60.3	58
$Alpha_1$		3.0	3
$Alpha_{1-2}$	2.0	7.6	7
$Alpha_2$	5.6		
$Alpha_3$	0.8	12.4	13
Beta	7.4		
Gamma (very fast)	4.2		
Gamma (fast)	1.9	16.5	19
Gamma	14.6		

* Electrophoretic distribution of chromatographic components as illustrated in Figure 2.
† Whole serum electrophoretic analysis.

chromatogram illustrated in Figure 2 with the results of whole serum analysis. Good agreement is evident, suggesting that no major change in electrophoretic properties was produced by exposure of the serum proteins to the chromatographic procedure.

The sequence of serum protein elution is similar to that reported previously with a more complex procedure (2). Gamma globulins are the first proteins to appear in the effluent fractions. A high peak containing most of the gamma globulin is seen early in the chromatogram. However, gamma globulins continue to be removed from the column in succeeding fractions and are characterized by a gradually increasing electrophoretic mobility (Figure 3). Finally a small amount of gamma globulin of low mobility is seen again in the latter portion of the chromatogram. The progressive elution of electrophoretically faster-moving proteins extending into the "slow beta" electrophoretic region emphasizes the existence of a spectrum of electrical properties among the proteins in the gamma globulin region.

The beta globulins are divided into at least three components by the chromatographic procedure, and the alpha globulins are divided into at least seven components (Figure 2).

Albumin emerges approximately midway through the elution procedure. The major portion of the albumin is obtained in a relatively narrow portion of the effluent volume. However, albumin continues to be eluted from the column in progressively decreasing amounts.

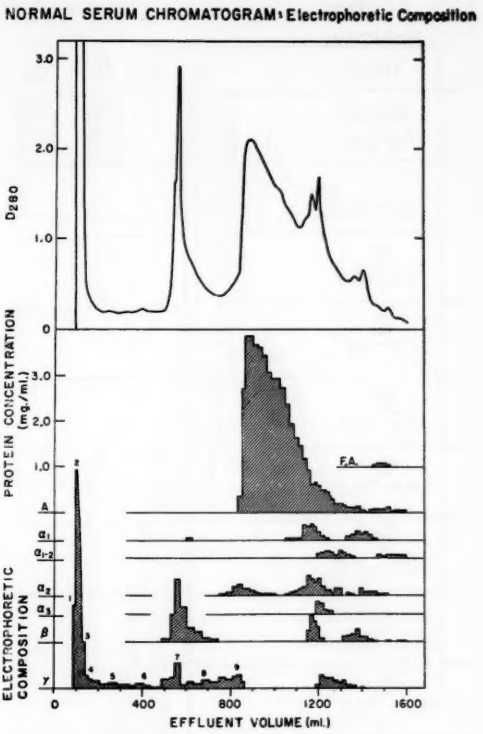

FIG. 2. NORMAL SERUM CHROMATOGRAM: ELECTROPHORETIC COMPOSITION

Chromatographic distribution of protein (D_{280}) is represented in the upper half of the figure. The distribution of electrophoretic components is illustrated below. In each of the 66 chromatogram fractions the total protein content was measured by the biuret technique and the amount of each electrophoretic component was determined on the basis of the relative quantity found on paper electrophoresis. Each electrophoretic category (*e.g.*, A, α, α 1–2) is plotted from its own baseline. The scale of protein concentration is presented only from the albumin baseline but applies to all of the categories. F. A. refers to a component with an electrophoretic mobility faster-than-albumin. Twenty ml. of normal serum and the standard chromatographic procedure employing 16 Gm. of adsorbent, a flow rate of 20 to 25 ml. per hour., effluent collected in 4 ml. fractions, and gradient elution with 1,000 ml. of 0.01 M pH 8 sodium phosphate buffer in the round flask and 500 ml. of 0.30 M monosodium phosphate in the Erlenmeyer flask were utilized.

Among the last components to be eluted from the column is one that has the interesting property of migrating faster than albumin in an electric field (Figure 2). This component comprises

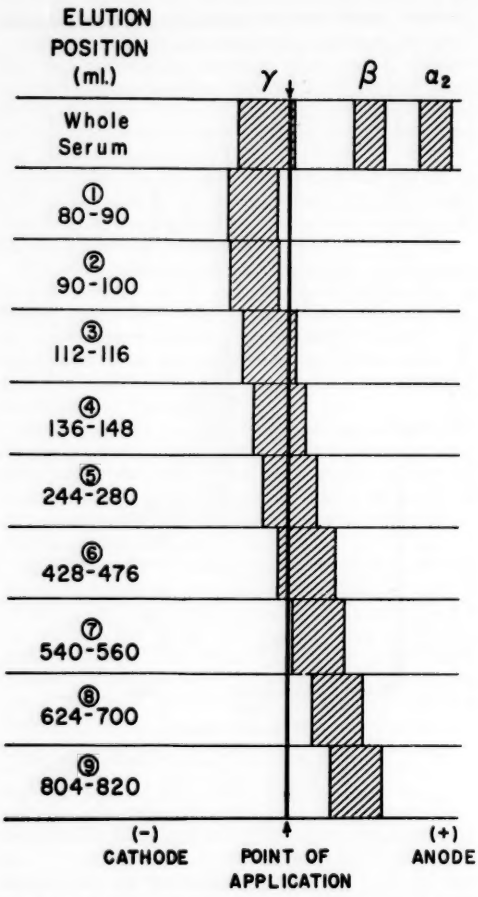

FIG. 3. ELECTROPHORETIC CHARACTERISTICS OF SERIAL FRACTIONS ELUTED IN THE FIRST PART OF THE CHROMATOGRAM ("GAMMA" GLOBULINS)

The fraction sites are identified in Figure 2 as 1, 2, 3, *et cetera*. Electrophoresis was carried out on approximately 0.1 mg. of protein from each fraction, and the sequence of samples in the electrophoresis cell was randomized so that mobility progression could not be attributed to a procedural artifact.

about 0.1 to 0.2 per cent of the total protein applied to the column.

Colored protein bands are readily apparent on the column during chromatography, and three, sometimes four, pigmented regions are detectable in the effluent fractions. A pink color is noted in the 500 to 600 ml. region (Figure 2), corresponding to the distribution of the iron-binding protein. If the serum contains appreciable hemoglobin, a red pigment will be detectable in the effluent just prior to albumin elution, suggesting the location there of the hemoglobin-haptoglobin complex (16, 5). Yellow pigments are seen in regions containing albumin. A blue component emerges in the region of 1,400 ml. and corresponds to the location of ceruloplasmin (2).

The pH and phosphate molarity of the effluent solutions are illustrated in Figure 4 for a 16 Gm. adsorbent chromatogram (20 ml. normal serum). These results provide a general picture of the conditions for protein adsorption and elution during chromatography, since a relatively fixed elution schedule was employed throughout these studies.[5] A sharp fall in pH is noted to occur coincident with the appearance of the second major protein peak. This association is characteristic of the chromatographic procedure employed in these experiments. The conditions determining protein adsorption and elution from DEAE-cellulose columns have been discussed elsewhere (1, 17, 18).

Representative results of the chromatography of 10, 2, and 1 ml. serum samples are illustrated in Figure 5. Also included in this figure are the results of electrophoretic examination of serial

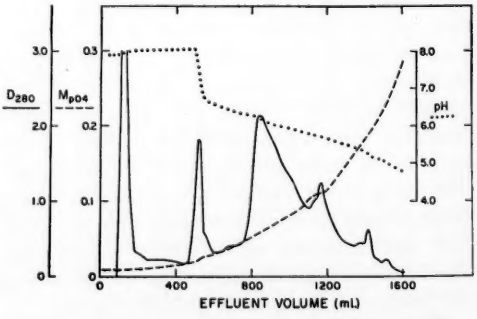

FIG. 4. SERUM CHROMATOGRAM: pH AND MOLARITY CHANGES IN THE EFFLUENT

Normal serum (20 ml.) was chromatographed. Protein distribution is indicated by the solid line, pH by the dotted line, and phosphate molarity by the broken line.

[5] In several of the chromatograms the relative level of the bottoms of the round and Erlenmeyer flasks was altered by a few millimeters with slight shift in component elution volume. This is most readily apparent with albumin. However, these minor changes are not thought to have significantly altered relative elution volumes.

subdivisions of the 1 ml. serum chromatogram. Although component resolution appears to be reduced with the smaller columns, this is due at least partially to inability to reduce proportionately the volume of effluent fractions. Actually, the first alpha-2 component is better separated from albumin under the conditions employed on the 3 and 2 Gm. adsorbent columns.

For a 20 ml. serum sample, about three days were required for column elution, and the entire procedure from the drawing of the blood sample to determination of the protein concentration of the final chromatographic fraction can be carried out in five days by one person. For the chromatography of 2 and 1 ml. serum samples only 24 hours were needed for column elution and several samples could be chromatographed concurrently by one person.

Although the detail of resolution is reduced in the small scale chromatograms, unless the individual elution fractions are made inordinately small, a considerable saving in time and sample can be achieved by such modified procedures.

The conditions for chromatography utilized here are relatively mild, employing buffers with a pH range of 8.0 to 4.5 and molarity from 0.01 to 0.30 M. Several specific serum proteins, alkaline phosphatase, iron-, thyroxin-, and B_{12}-binding protein, retained their specific functions and do not appear to have been altered by the chromatographic procedure.

Protein-bound carbohydrate

Most serum proteins contain some carbohydrate although the relative amount varies markedly among individual proteins (19). Protein-bound hexose has been measured in the effluent of eight serum chromatograms, and the findings in a representative normal chromatogram are illustrated in Figure 6.

At least five distinct peaks of hexose distribution are evident. The initial chromatographic peak, composed solely of gamma globulins, contained 1.2 per cent hexose, which agrees well with published data for the gamma globulins (20). The remaining carbohydrate peaks represent the composite hexose content of several electrophoretically distinct protein components. Further studies are underway to elucidate the distribution

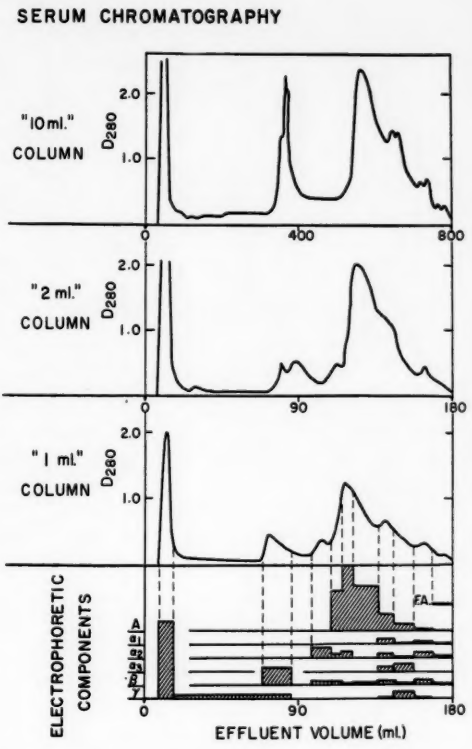

FIG. 5. CHROMATOGRAPHY OF 10, 2, AND 1 ML. SERUM SAMPLES

Aliquots of a single sample obtained from a normal individual were employed. The conditions of chromatography are outlined in Table I. For the 2 ml. serum sample an eluting system of 110 ml. of 0.01 M pH 8 phosphate buffer in the round flask, 55 ml. of 0.30 M monosodium phosphate in the Erlenmeyer flask, and a 3 Gm. adsorbent column were utilized. Electrophoretic analysis of the 1 ml. serum chromatogram is also illustrated.

of hexose among these proteins in normal and pathologic sera.

In a previous study using related chromatographic procedures (2) orosomucoid and a high hexosamine content were found in a region corresponding to the peak at 1,370 ml. in Figure 6. Hexosamine was also found in a region approximating the 550 ml. peak. The present studies emphasize the distribution of hexose among many serum components. Gamma globulin and at least four other chromatographic regions contribute to the total serum level. Since albumin is reported

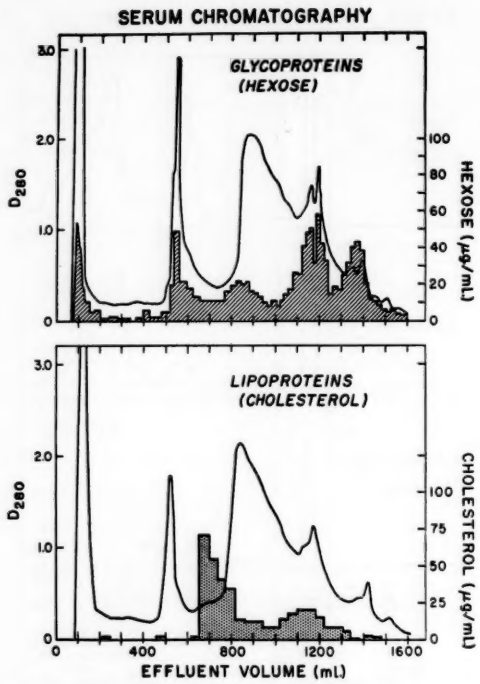

FIG. 6. PROTEIN-BOUND CARBOHYDRATE (HEXOSE) AND CHOLESTEROL DISTRIBUTION

Protein distribution (D_{280}) is outlined by the solid line, and hexose and cholesterol distribution by the shaded areas. The 20 ml. serum samples employed for these chromatograms were obtained from two normal male donors.

to be free of carbohydrate (19), it should be noted that, although the carbohydrate level at the site of albumin elution is quite low (0.3 per cent), the finding of any carbohydrate here suggests the presence of protein components other than albumin which were not detected on electrophoretic analysis.

Protein-bound cholesterol

The chromatographic distribution of cholesterol-containing proteins is also illustrated in Figure 6. These lipoproteins are seen to be distributed among several regions in the chromatogram. More than one protein is present in these regions, and localization of the cholesterol with respect to electrophoretic and other categories remains to be done.

Siderophilin

Plasma iron is normally bound to a beta globulin referred to as siderophilin, iron-binding protein, or transferrin (21). The location of siderophilin in the chromatogram was ascertained by addition of radioactive iron to normal serum prior to chromatography.[6] The similarity of distribution of radioactive iron and protein throughout the peak at 500 to 600 ml. is illustrated in Figure 7.

Electrophoretic examination of the chromatographic region containing the siderophilin revealed it to be composed primarily of a beta globulin (85 per cent), consistent with the known electrophoretic character of siderophilin. However, this region also contained a fast gamma electrophoretic component that comprised 15 per cent of the protein present. This "gamma" globulin constituted a variable minor proportion of the protein eluted in the 500 to 600 ml. region by the chromatographic procedure employed throughout this study. Recent refinements in technique have further improved the separation of siderophilin from gamma globulin (5). It should be noted that with the present chromatographic procedure the siderophilin is separated from much of the beta globulin which emerges later in the eluate.

Vitamin B_{12}-binding protein

The vitamin B_{12} present in serum is largely bound to the serum proteins. In chronic myelocytic leukemia the serum B_{12} level may be markedly elevated (22). Chromatography of serum from a patient with chronic myelocytic leukemia and a serum B_{12} level 20 times normal is illustrated in Figure 7. The B_{12} activity is almost entirely confined to a single region in the latter third of the chromatogram. This region was further fractionated by electrophoretic techniques and the B_{12} activity was located in an alpha globulin fraction. This has been confirmed in additional studies on similar sera (13).

The finding of B_{12} in an alpha globulin fraction is in agreement with observations made by paper electrophoresis alone (23, 24). The use of chromatographic techniques in the present work has

[6] For assistance in this experiment we are greatly indebted to Drs. A. L. Schade, R. C. Woodworth, and J. M. Stengle.

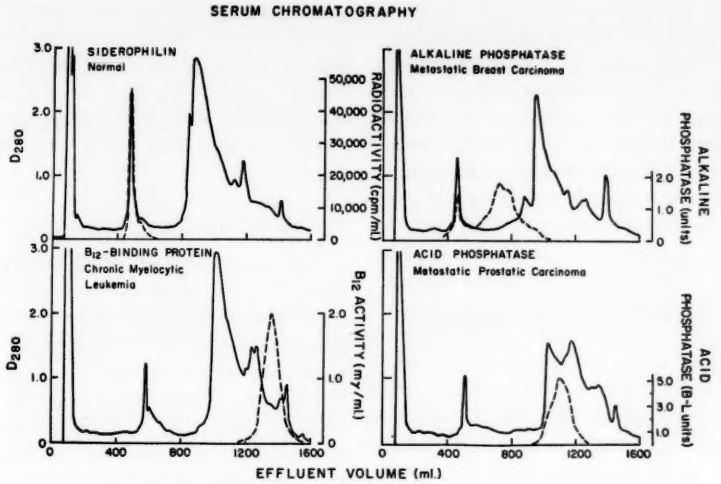

FIG. 7. CHROMATOGRAPHIC LOCATION OF SIDEROPHILIN, B_{12}-BINDING PROTEIN, ALKALINE AND ACID PHOSPHATASES

Protein distribution is represented by the solid line and special activities by broken lines. Amounts of dialyzed serum applied to the columns: siderophilin, 20 ml., with total protein of 6.5 Gm. per cent; B_{12}-binding protein, 18 ml., with total protein of 6.6 Gm. per cent and B_{12} content of 12.0 millimicrograms per ml.; alkaline phosphatase, 20 ml., with total protein of 4.5 Gm. per cent and 22.5 units alkaline phosphatase per ml.; acid phosphatase, 19 ml., with total protein of 4.3 Gm. per cent and 43.2 B-L acid phosphatase units per ml.

permitted further separation of the B_{12}-binding protein from several of the components which contribute to the alpha globulins.[7]

Alkaline and acid phosphatases

Alkaline and acid phosphatases, enzymatic activities normally present in the serum in small amounts, may be markedly increased with disease. Chromatography of serum having a high alkaline phosphatase activity, obtained from a patient with carcinoma of the breast and metastases to liver and bone, revealed the alkaline phosphatase activity to be largely distributed in two peaks (Figure 7). The possibility that these peaks might represent two different enzymes has been explored and confirmed in further studies carried out in collaboration with Dr. A. C. Peacock (25).

Chromatography of a serum with elevated acid phosphatase activity, obtained from a patient with metastatic prostatic carcinoma, is also illustrated in Figure 7. The distribution of acid phosphatase activity is seen to differ from that of alkaline phosphatase. This observation adds additional evidence indicating the separate identity of these enzymes.[8]

Thyroxin-binding protein

Circulating thyroxin has been found to be largely bound to a specific alpha globulin, referred to as the thyroxin-binding protein (26). The chromatographic location of the thyroxin-binding protein was determined by addition of an excess of radioiodine-labeled thyroxin to normal serum *in vitro* prior to dialysis and chromatography.[9] Electrophoretic analysis of this labeled serum before chromatography revealed that the alpha thyroxin-binding protein contained 40 per cent of

[7] This study of the B_{12}-binding protein was conducted in collaboration with Drs. R. S. Mendelsohn and D. M. Watkin.

[8] The phosphatase observations were carried out in collaboration with Dr. A. C. Peacock.

[9] This study was carried out in collaboration with Dr. Jacob Robbins.

the radioactivity and was saturated with thyroxin. The remaining 60 per cent of the radioactivity was associated with albumin. The results illustrated in Figure 8 demonstrate one principal peak of radioactivity. Further testing of 10 effluent fractions obtained throughout this region demonstrated that the radioactivity was nondialyzable and that on electrophoresis it was primarily confined to an alpha globulin fraction. Very little of the radioactivity was associated with albumin, and on completion of chromatography a considerable amount of radioactivity remained on the column. This material was eluted with 0.5 N NaOH and found to be ultrafiltrable, and is presumed to have been thyroxin that originally was associated with albumin. The small peak of radioactivity seen near the end of the chromatogram was found on electrophoresis to be associated with the faster-than-albumin components of this chromatographic region. It is not known whether this component is thyroxin or one of the nonthyroxin contaminants of the radioactive thyroxin preparation.

The elution schedule employed for this chromatogram differed from that utilized throughout the remainder of this study and achieved a moderate spreading of the components which compose the latter half of the usual chromatogram. It seems unlikely that the relative chromatographic location of the thyroxin-binding protein was altered by this modification in procedure. The finding of thyroxin bound to an alpha-globulin confirmed results obtained by electrophoretic techniques alone (15), and, in addition, a further separation of thyroxin-binding protein from a portion of the other alpha globulin components was achieved.

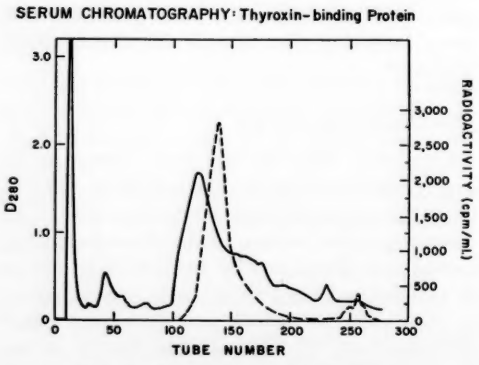

FIG. 8. THYROXIN-BINDING PROTEIN

Chromatogram obtained after addition of radioiodine-labeled thyroxin to normal serum. Protein distribution is indicated by the solid line and radioactivity by the broken line. The elution schedule employed for this chromatogram was not the usual one and is outlined under the Methods section.

Radioiodine-labeled albumin

Radioactive iodine-labeled human serum albumin (RISA) was added to normal serum prior to dialysis and chromatography.[10] This iodinated albumin is available commercially and is commonly used in this country for observations on the physiology of human serum albumin (27). However, as is seen in Figure 9, the chromatographic distribution of the radioactive albumin did not correspond to that of the bulk of the serum albumin.

Further examination of the effluent revealed that the radioactivity was not dialyzable and was associated only with albumin when electrophoresis was performed on the regions of the chromatogram containing radioactivity. The results indicate that the radioactive albumin was not representative of the native serum albumin, at least in

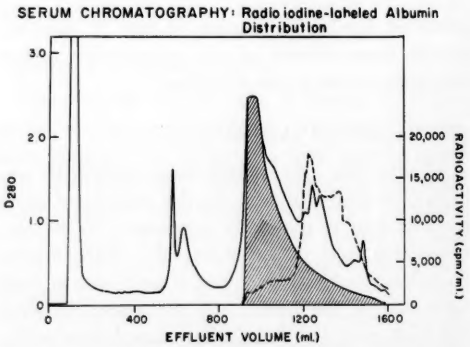

FIG. 9. RADIOIODINATED ALBUMIN

One mg. of iodinated albumin (20 μc. of I^{131}) was added, prior to dialysis, to 27 ml. of fresh normal human serum containing 961 mg. of albumin, and 20 ml. of the dialyzed serum was chromatographed. The protein distribution in the chromatogram is indicated by the solid line, the albumin distribution by the shaded area, and the distribution of radioactivity (iodinated albumin) by the broken line.

[10] This study was carried out in collaboration with Dr. J. L. Steinfeld.

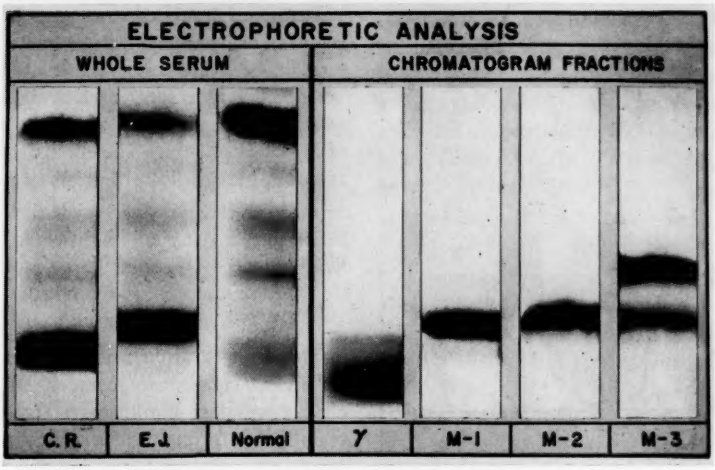

FIG. 10. PAPER ELECTROPHORETIC PATTERNS OF MULTIPLE MYELOMA SERA AND CHROMATOGRAM FRACTIONS

The paper electrophoretic patterns are arranged so that albumin is seen at the upper (anodal) end of the normal serum strip and gamma globulin at the lower end. Electrophoretic analysis of the first four E. J. chromatogram peaks are included.

regard to behavior during column chromatography. This difference is of interest because of the large amount of information obtained in recent years on the biological behavior of iodinated albumin and the general inference from these studies as to the biological characteristics of native albumin.

The present studies do not indicate whether the difference in chromatographic distribution of the native serum albumin and the iodinated albumin resulted from changes induced during preparation or during "pasteurization" of the albumin, by selective iodination of a distinctive group of albumin molecules, by the presence of iodine on the tyrosine residues of albumin, by the oxidation procedure used to achieve iodination, or by the effects of radioactivity on the albumin molecules. Further studies are underway to elicit this information.

Myeloma protein

Sera from two patients with multiple myeloma were selected for chromatographic analysis. Both sera on electrophoretic examination demonstrated large amounts of a homogeneous protein having a gamma mobility (Figure 10). The chromatographic results, illustrated in Figure 11, demonstrate that in both instances the abnormal protein emerged from the column near the region where most of the normal gamma globulin appear. No abnormal protein was discerned in the later parts of the chromatogram. A diminished peak in the albumin region corresponded to the lowered serum albumin seen on electrophoresis. It is noteworthy that the myeloma protein patterns of the C. R. and E. J. serum chromatograms suggest the presence of several components. This is particularly evident in the E. J. serum. Chromatographic analysis was repeated on both sera with similar results. Serial electrophoretic examination of the myeloma regions from the E. J. chromatogram is recorded in Figure 10. There was no evidence of electrophoretic distinction among the several chromatographic peaks.

Of particular interest in the serum chromatogram of E. J. was the observation of a small protein peak at the site of normal gamma globulin elution. Electrophoretic examination of this peak revealed a qualitatively normal gamma globulin pattern without evidence of any abnormal component (Figure 10). The adjacent M peak, however, was found to consist of electrophoretically

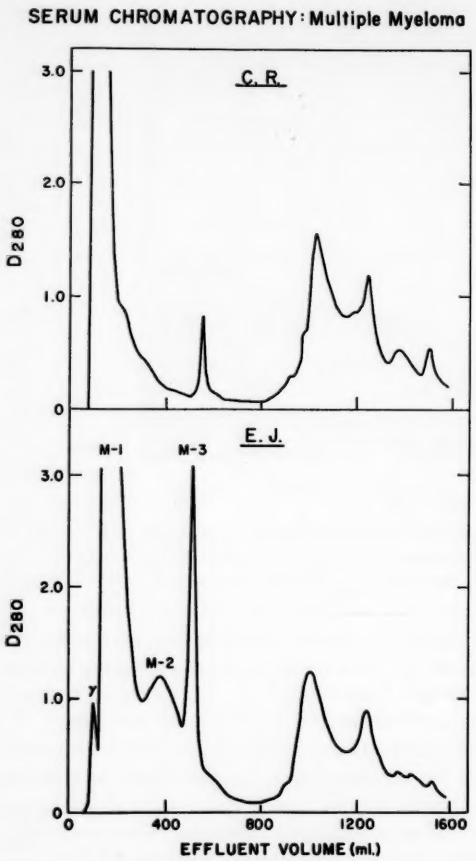

FIG. 11. MULTIPLE MYELOMA

Sera from two subjects with multiple myeloma were chromatographed in the usual way. The large amount of protein eluted in the initial portion of the chromatogram could not be included within the limits of this scale.

homogeneous protein of narrow distribution that was characteristic of the myeloma protein seen in the whole serum. Gamma globulin was not evident in this chromatographic component. The chromatographic separation of "normal" gamma globulin from myeloma protein in the E. J. serum was improved on further chromatographic study by using a starting buffer of lower molarity (0.005 M). In the C. R. serum the myeloma protein began to emerge at the same site as normal gamma globulin and no distinction could be made chromatographically between normal gamma globulin and the initial myeloma protein peak.

Decreased levels of gamma globulin have been reported previously in patients with multiple myeloma (28). However, these observations have been restricted to sera in which the electrophoretic gamma region was not obscured by a large amount of myeloma protein. The chromatographic results reported here indicate that anion-exchange cellulose chromatography may be useful in certain instances for the evaluation of the serum proteins of patients with multiple myeloma.

DISCUSSION

The demonstration that the serum proteins can be divided into many components by anion-exchange cellulose chromatography suggests many applications of this technique in the study of proteins (2). The simplified method presented here has preserved most of the resolving capacity of the DEAE-cellulose column while making the procedure more suitable for clinical studies. The chromatographic and analytic results reported here are in essential agreement with the earlier observations on the application of ion-exchange cellulose chromatography to human serum (2).

Fractionation of the serum electrophoretic categories is one of the most interesting characteristics of DEAE-cellulose chromatography (2). Subdivision of alpha and beta globulins is readily apparent. In the present study fractionation of the serum glycoproteins and lipoproteins has been demonstrated. The beta iron-binding globulin and the beta lipoproteins have been separated chromatographically. The determination of siderophilin distribution by means of a radioactive iron preparation also confirms results obtained by a spectrophotometric technique (2). Among the alpha globulin subdivisions the thyroxin-binding protein, vitamin B_{12}-binding protein and ceruloplasmin have been identified. Acid and alkaline phosphatases in pathologic serum have also been separated by Boman and Westlund (4) who employed anion-exchange chromatography on columns of Dowex-2 resin. This resin, however, has a low capacity for proteins when compared with DEAE-cellulose (1).

Albumin also appears to be fractionated by anion-exchange cellulose chromatography. Previous immunologic and electrophoretic studies of albumin fractions obtained by chromatographic

separation suggested differences between the albumins of several fractions (2). The chromatographic distribution of the radioiodinated albumin preparations that have been tested differed markedly from the distribution of the bulk of native serum albumin.

The composition and significance of the faster-than-albumin component is unknown at present. Hoch and Chanutin observed such a component, and in about the same concentration, on electrophoresis of normal undialyzed plasma in 0.1 M NaCl at pH 8 (29).

Tiselius, Hjerten, and Levin (3) have reported fractionation of human gamma globulin and of bovine serum albumin on calcium phosphate columns, and Porter utilized celite columns to fractionate the immune proteins among the gamma globulins (30). Cation-exchange cellulose columns have also been employed to fractionate human gamma globulin (31). These reports and the findings with anion-exchange cellulose chromatography serve to reemphasize the heterogeneity of materials normally present in the serum electrophoretic compartments.

Chromatographic analysis has been performed on many individual normal sera and also on sera obtained from patients with a variety of diseases. The chromatographic patterns of the pathologic sera were frequently abnormal. Chromatograms of pathologic sera obtained from patients with chronic myelocytic leukemia, carcinoma of the breast, carcinoma of the prostate and multiple myeloma are presented. In several instances the initial (gamma) peak was increased or decreased, the peaks containing siderophilin and albumin were decreased, and the regions containing ceruloplasmin and the alpha glycoproteins were increased.

These findings, although preliminary, suggest that anion-exchange cellulose chromatography may have considerable value as an analytic procedure, in addition to its evident usefulness in protein fractionation. The potentialities of chromatography as an analytic procedure are perhaps best illustrated in the present report by the observations on radioiodinated albumin and myeloma protein distribution. Whether myeloma serum will yield characteristic chromatograms distinctly different from those found with sera containing markedly elevated gamma globulins of other etiology remains to be seen. However, the demonstration that in certain instances the myeloma protein may be separable from the "normal" gamma globulins does indicate that anion-exchange cellulose chromatography may be useful for the quantification of the "normal" gamma globulins present in this disease.

SUMMARY

A modified anion-exchange (DEAE-) cellulose chromatographic procedure for the separation and characterization of serum proteins is described. This procedure has been applied to serum samples of 20, 10, 2, and 1 ml.

The chromatographic fractions of normal serum were characterized by electrophoresis and by carbohydrate and cholesterol content. The usual serum electrophoretic components have been subdivided into several fractions by this chromatographic procedure, and a faster-than-albumin component has been regularly uncovered. The chromatographic distribution of siderophilin (iron-binding protein), thyroxin-binding protein, B_{12}-binding protein, and acid and alkaline phosphatases was determined. These components were separated on the chromatogram from many proteins of similar electrophoretic mobility. The chromatographic distribution of radioiodinated human serum albumin was found to differ significantly from the distribution of native serum albumin.

Chromatograms of serum obtained from patients with chronic myelocytic leukemia, carcinoma of the breast, prostatic carcinoma, and multiple myeloma are presented. Several of these differed markedly from the normal chromatograms. In one instance the myeloma protein appeared to be separable from most of the normal gamma globulin. The significance of these observations is discussed.

ACKNOWLEDGMENTS

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REFERENCES

1. Peterson, E. A., and Sober, H. A. Chromatography of proteins. I. Cellulose ion-exchange adsorbents. J. Amer. chem. Soc. 1956, **78**, 751.

2. Sober, H. A., Gutter, F. J., Wyckoff, M. M., and Peterson, E. A. Chromatography of proteins. II. Fractionation of serum protein on anion-exchange cellulose. J. Amer. chem. Soc. 1956, **78**, 756.
3. Tiselius, A., Hjerten, S., and Levin, O. Protein chromatography on calcium phosphate columns. Arch. Biochem. 1956, **65**, 132.
4. Boman, H. G., and Westlund, L. E. Protein chromatography on an anion-exchange resin. Arch. Biochem. 1956, **64**, 217.
5. Peterson, E. A., and Sober, H. A. Personal communication.
6. Gornall, A. G., Bardawill, C. J., and David, M. M. Determination of serum proteins by means of the biuret reaction. J. biol. Chem. 1949, **177**, 751.
7. Lever, W. F., Gurd, F. R. N., Uroma, E., Brown, R. K., Barnes, B. A., Schmid, K., and Schultz, E. L. Chemical, clinical, and immunological studies on the products of human plasma fractionation. XL. Quantitative separation and determination of the protein components in small amounts of normal human plasma. J. clin. Invest. 1951, **30**, 99.
8. Williams, F. G., Jr., Pickels, E. G., and Durrum, E. L. Improved hanging-strip paper electrophoresis technique. Science 1955, **121**, 829.
9. Jencks, W. P., Jetton, M. R., and Durrum, E. L. Paper electrophoresis as a quantitative method. Biochem. J. 1955, **60**, 205.
10. Shetlar, M. R., and Masters, Y. F. Use of thymol-sulfuric acid reaction for determination of carbohydrates in biological material. Analyt. Chem. 1957, **29**, 402.
11. Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E. A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. biol. Chem. 1952, **195**, 357.
12. Schade, A. L., Oyama, J., Reinhart, R. W., and Miller, J. R. Bound iron and unsaturated iron-binding capacity of serum; rapid and reliable quantitative determination. Proc. Soc. exp. Biol. (N. Y.) 1954, **87**, 443.
13. Mendelsohn, R. S., Watkin, D. M., Horbett, A. P., and Fahey, J. L. Identification of the vitamin B_{12} binding protein in the sera of normals and of patients with chronic myelocytic leukemia. In preparation.
14. Bessey, O. A., Lowry, O. H., and Brock, M. J. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. J. biol. Chem. 1946, **164**, 321.
15. Robbins, J. Reverse-flow zone electrophoresis. A method for determining the thyroxine-binding capacity of serum protein. Arch. Biochem. 1956, **63**, 461.
16. Jayle, M. F., Boussier, G., and Badin, J. Électrophorèse de l'haptoglobine et de son complexe hémoglobinique. Bull. Soc. Chim. biol. (Paris) 1952, **34**, 1063.
17. Moore, S., and Stein, W. H. Column chromatography of peptides and proteins. Advanc. Protein Chem. 1956, **11**, 191.
18. Sober, H. A., and Peterson, E. A. Chromatography of proteins and nuclei acids *in* Ion Exchangers in Organic and Biochemistry, C. Calmon and T. R. E. Kressman, Eds. New York, Interscience Publishers, 1957, p. 318.
19. Hughes, W. L. Interstitial proteins: The proteins of blood plasma and lymph *in* The Proteins, H. Neurath and K. Bailey, Eds. New York, Academic Press Inc., 1954, vol. II, p. 663.
20. Müller-Eberhard, H. J., and Kunkel, H. G. The carbohydrate of γ-globulin and myeloma proteins. J. exp. Med. 1956, **104**, 253.
21. Laurell, C.-B. Plasma iron and the transport of iron in the organism. Pharmacol. Rev. 1952, **4**, 371.
22. Beard, M. F., Pitney, W. R., and Sanneman, E. H. Serum concentrations of vitamin B_{12} in patients suffering from leukemia. Blood 1954, **9**, 789.
23. Pitney, W. R., Beard, M. F., and Van Loon, E. J. Observations on the bound form of vitamin B_{12} in human serum. J. biol. Chem. 1954, **207**, 143.
24. Heinrich, H. C., and Erdmann-Oehlecker, S. Der Vitamin B_{12}-Stoffwechsel bei Hämoblastosen. II. Die intravitale Bindung (Transport) der B_{12}-Vitamine an die Serumproteinfraktionen bei Hämoblastosen. Clinica chim. Acta 1956, **1**, 311.
25. Peacock, A. C., and Fahey, J. L. Demonstration of two alkaline phosphatase activities in normal and pathologic sera. In preparation.
26. Robbins, J., and Rall, J. E. Interaction of thyroid hormones and proteins in biological fluids *in* Recent Progr. Hormone Res. In press.
27. The role of I^{131} labeled proteins in biology and medicine, S. P. Masouredis, Ed. Ann. N. Y. Acad. Sci. 1957, **70**, pp. 1–152.
28. Adams, W. S., Alling, E. L., and Lawrence, J. S. Multiple myeloma: Its clinical and laboratory diagnosis with emphasis on electrophoretic abnormalities. Amer. J. Med. 1949, **6**, 141.
29. Hoch, H., and Chanutin, A. Electrophoretic studies on fast moving components of human serum. J. biol. Chem. 1953, **200**, 241.
30. Porter, R. R. Fractionation of rabbit gamma-globulin by partition chromatography. Biochem. J. 1955, **59**, 405.
31. Sober, H. A., and Peterson, E. A. Chromatography of proteins on modified cellulose. Vox Sang. (Basel) 1957, **2**, 62.

EFFECTS OF HIGH SPINAL ANESTHESIA ON CEREBRAL CIRCULATION AND METABOLISM IN MAN[1,2]

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The effects of high spinal anesthesia on the general (1, 2), hepatic (3), and coronary (4) hemodynamics have been previously reported by our group. Because hypotension produced by high spinal anesthesia may be a potential hazard to the circulatory sufficiency of the brain, it was considered important to study the effects of high spinal anesthesia on the cerebral circulation and metabolism.

Studies by others have been concerned with the effects of differential spinal block on the cerebral circulation of hypertensive individuals (5) and the effect of hypotensive drugs on the cerebral circulation of aged persons (6) and hypertensives (7). The majority of patients in this study are normotensive, but the effects of high spinal anesthesia on a small group of hypertensives is included. The anesthetic level is higher and the amount of spinal anesthetic agent employed is larger than in studies reported by others, simulating the dosage used in producing surgical high spinal anesthesia, although surgical procedures were not undertaken in these subjects.

MATERIAL AND METHODS

Nineteen patients were selected from the medical wards. Of these, 15 were normotensive and free of cardiopulmonary disease, and 4 were hypertensive. Following an overnight fast and morning sedation (pentobarbital sodium, 0.1 Gm.) the patients were transported to the cardiovascular laboratory, where the ambient temperature was maintained between 23 and 24° C. In some instances a No. 6-8 F Goodale birds-eye catheter was introduced via a medial arm vein into the jugular bulb and the position of the tip checked repeatedly by fluoroscopic examination. In other instances direct puncture of the jugular bulb was employed. Blood samples were then drawn for basal determination of cerebral blood flow, arterial and jugular venous blood gases, pH, glucose, lactate, and pyruvate. Following the basal determinations, one group of 13 patients (age range, 22 to 60; average, 41.6 years) was given a spinal anesthetic of 150 to 200 mg. of procaine by barbotage. In all instances the arbitrary criteria previously described for high spinal anesthesia (1, 2) were met and confirmed by plethysmographic (8) demonstration of increased finger blood flow. Thirty minutes after the administration of the anesthetic all studies were repeated. A second group of six patients (age range, 44 to 66; average, 56 years) served as "double controls." Thirty minutes after the initial basal study a second set of determinations was performed without the administration of the anesthetic. In all instances patients were maintained in total head-down body tilt of 5 degrees. Vasopressor drugs were not given at any time.

Blood oxygen contents, carbon dioxide contents and pH determinations were performed in duplicate, and checks required to 0.2 volume per cent for oxygen and CO_2 and to 0.01 pH unit. Jugular venous and brachial arterial oxygen contents and arterial oxygen capacity were determined spectrophotometrically according to the method of Hickam and Frayser (9) and frequently spot-checked by simultaneous gasometric analysis. Carbon dioxide content of arterial blood was determined by the manometric method of Van Slyke and Neill (10) and arterial blood pH readings were obtained by use of the Cambridge glass electrode potentiometer at room temperature, and corrected to body temperature (11). Values for pCO_2 were obtained from the nomogram of Singer and Hastings (12). Glucose (13), lactate (14), and pyruvate (15) determinations were done in duplicate on arterial and venous blood.

Cerebral blood flow (CBF) was determined by the manometric technique of Kety and Schmidt (16) and expressed as ml. per 100 Gm. of brain tissue per minute. Cerebral oxygen consumption (CMR O_2) was calculated as the product of CBF and the arterial-jugular venous (A-VO_2) oxygen difference. This is expressed in terms of ml. per 100 Gm. of brain tissue per minute. Cerebral glucose consumption (CMR gl) was similarly calculated by using the arterial-jugular venous glucose (A-Vgl) difference and is expressed as mg. per 100 Gm. per minute.

The brachial arterial pressure was measured through a No. 19 indwelling arterial needle, transduced via Statham strain gauges, amplified by a Brush D.C. amplifier,

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and recorded on the Brush multi-channel oscillograph. Mean arterial blood pressures (MABP) were obtained by planimetric integration of the contours recorded during two successive respiratory cycles.

Cerebrovascular resistance (CVR) was calculated by the formula

$$CVR = \frac{\text{mean arterial blood pressure}}{\text{CBF per 100 Gm. per minute}},$$

and expressed as mm. Hg per ml. per 100 Gm. per minute. In calculating the mean arterial blood pressure, the readings taken prior to and immediately after the flows were averaged.

RESULTS

The data are presented in Tables I through III. The data have been treated statistically, although in the case of the hypertensives the small number of observations precluded such analysis.

Mean arterial blood pressure

The MABP in the normotensive group fell from 93 to 63 mm. Hg during high spinal anesthesia. The decrease in mean blood pressure for the four hypertensives was from 158 to 79 mm. Hg. In the double control group the change was from 100 to 106 mm. Hg. The marked decrease in the normotensive spinal anesthesia group is highly significant when compared to the variation in the double controls.

The cerebral blood flow

The mean CBF in the normotensive group did not change significantly during high spinal anesthesia. The prespinal value was 45 ml. per 100

TABLE I

*Cerebral hemodynamics and blood gases in high spinal anesthesia**

	Normotensives													
Patient	F. D.		J. R.		B. M.		C. T.		M. L.		S. K.		M. B.	
Diagnosis	Chronic alcoholism		Pneumonia, recovered		Pneumonia, recovered		Chronic alcoholism		Pyelo-nephritis, recovered		General paresis, treated		Barbiturate intoxication, recovered	
Sex	M		M		M		M		F		M		F	
Age	22		38		26		60		26		42		51	
Spinal level	T_2–T_3		T_3		T_2(R)–T_3(L)		T_2–T_3		T_1		T_2		T_2	
	C	E	C	E	C	E	C	E	C	E	C	E	C	E
Art. O_2, vol. %	9.6	10.6	18.5	18.4	18.9	19.4	10.6	10.5	13.1	12.4	17.2	16.2	16.0	16.0
Jug. Ven. O_2, vol. %	3.1	4.0	12.7	12.6	13.7	12.5	4.9	3.8	6.8	6.9	8.4	8.3	10.2	10.3
A-V O_2, vol. %	6.5	6.6	5.8	5.8	5.2	6.9	5.7	6.7	6.3	5.5	8.8	7.9	5.8	5.7
O_2 Cap., vol. %	10.6	11.6	20.2	19.4	19.8	19.7	11.8	11.6	13.9	13.3	18.2	17.6	17.6	17.3
Art. Sat., %	91	91	92	95	96	99	90	90	94	93	95	93	91	91
Art. CO_2, vol. %	39.4	41.9	47.0	46.4	46.3	48.0	45.5	45.3	40.1	40.2	39.0	39.7	44.0	42.4
Art. pH	7.41	7.35	7.41	7.40	7.34	7.35	7.38	7.42	7.40	7.40	7.41	7.42	7.42	7.40
Art. pCO_2, mm. Hg	31	37	40	40	46	46	38	35	33	33	33	32	35	35
Pulse	75	60			83	86	91	94	81	73	70	68	79	68
MABP, mm. Hg	92	62	92	66	100	66	99	59	69	55	98	61	86	72
CBF, ml./100 Gm./min.	37	41	58	60	55	49	31	29	50	59	46	49	41	49
CMR O_2, ml./100 Gm./min.	2.4	2.7	3.2	3.3	2.9	3.4	1.8	1.9	3.1	3.2	4.1	3.9	2.4	2.8
CVR	2.4	1.7	1.7	1.0	1.8	1.3	2.9	2.0	1.4	0.9	2.1	1.4	2.1	1.5
CMR gl., mg./100 Gm./min.	6.3	4.1	3.3	3.4	1.6	2.9	2.5	2.0	3.0	5.9	4.1	2.9	5.6	0.6

* Abbreviations: MABP = mean arterial blood pressure; CBF =cerebral arterial blood pressure; CMR O_2 = cerebral oxygen consumption; CVR = cerebral vascular resistance expressed as mm. Hg/ml./100 Gm./min.; CMR gl = cerebral glucose consumption.

† Signifies a statistically significant difference when compared with the double control group ($p = <0.01$).

TABLE I—*Continued*

Cerebral hemodynamics and blood gases in high spinal anesthesia

Normotensives—*continued*						Hypertensives									
M. S.		A. G.		Mean		M. B.		E. T.		J. H.		F. E.		Mean	
Pneumonia, recovered		Chronic pancreatitis				Essential hypertension		Essential hypertension		Essential hypertension		Essential hypertension			
M		F		6 male 3 female		F		F		M		F		1 male 3 female	
35		54		39.3		39		36		52		60		46.8	
T_3		T_2				T_1		T_1		T_2		T_2–T_4			
C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E
20.1	19.2	19.2	18.4	15.9	15.7	16.4	14.6	15.4	13.6	20.2	19.0	18.1	17.1	17.5	16.1
12.5	10.0	12.3	10.3	9.4	8.7	9.1	6.0	9.5	6.8	11.0	5.1	11.7	9.8	10.3	6.9
7.6	9.2	6.9	8.1	6.5	6.9	7.4	8.6	5.9	6.8	9.2	13.9	6.4	7.3	7.2	9.2
21.2	20.4	20.5	19.7	17.1	16.8	17.6	16.0	16.4	14.6			19.2	18.8	17.7	16.4
95	94	94	93	93	93	94	91	94	93			94	93	94	92
48.4	42.7	45.2	44.1	43.9	43.4	38.3	36.3			43.0	34.9	49.4	48.0	43.6	39.7
7.41	7.43			7.40	7.40	7.40	7.39	7.43	7.41	7.41	7.54	7.42	7.41	7.41	7.44
41	35			37	37	33	31			37	23	41	40	37	31
79	79	68	62	78	74	89	79	81	58	84	68	98	102	88	77
91	69	106	61	92.5	63.4†	124	59	157	110	151	48	199	98	158	79
37	38	46	41	44.6	46.1	42	34	46	44	60	32	38	40	46.5	37.5
2.8	3.5	3.2	3.3	2.9	3.1	3.1	2.9	2.7	3.0	5.5	4.3	2.4	2.9	3.4	3.3
2.5	1.8	2.3	1.5	2.1	1.5†	3.0	1.7	3.4	2.5	2.5	1.5	5.2	2.5	3.5	2.1
5.7	5.5	6.0	3.3	4.2	3.2	2.1	3.4			2.4	4.5				

Gm. per minute as compared to 46 ml. during high spinal study. Three of the four hypertensive patients showed a decrease in CBF during spinal anesthesia. The double control studies showed a variation in mean values from 45 ml. to 43 ml. per 100 Gm. per minute. When the variation in the mean CBF in the normotensive high spinal group is compared to the double control variations, the change is not statistically significant.

The cerebral oxygen consumption

There was no significant change in the mean CMR O_2 in the normotensive patients during high spinal anesthesia as compared to the prespinal value. The small group of hypertensives similarly showed no change in mean CMR O_2 during high spinal anesthesia. The mean CMR O_2 was also unchanged during the double control studies. When the variation in mean CMR O_2 values from prespinal to high spinal was compared to those noted in the double control groups, there was no significant difference.

The cerebral vascular resistance

There was a marked and statistically significant decrease in the mean CVR in the normotensive patients during high spinal anesthesia. The hypertensive patients showed a similar change. In double control studies there was a slight increase from 2.1 to 2.4 units; this change was not significant. The marked decrease in mean CVR in the normotensive patients during high spinal anesthesia was significant when compared to the variation in the double control group.

TABLE II

Cerebral hemodynamics and blood gases in double controls

Patient	W. O.		D. M.		J. C.		A. B.		V. P.		H. R.		Mean	
Diagnosis	Chronic alcoholism		Pneumonia, recovered		Cerebral arterio-sclerosis		Essential hypertension, uncomplicated		Chronic rheumatoid arthritis		Acute tonsillitis, recovered			
Sex	Male		Male		Male		Female		Male		Male		5 male 1 female	
Age	57		55		58		44		55		66		56	
	C	E	C	E	C	E	C	E	C	E	C	E	C	E
Art. O_2, vol. %	14.5	14.4	10.9	10.9	16.7	16.8	16.6	16.8	19.0	19.1	14.0	13.9	15.3	15.3
J. V. O_2, vol. %	9.6	7.8	5.9	5.8	9.5	9.5	10.6	10.7	12.1	12.4	7.1	7.0	9.1	8.9
A-V O_2, vol. %	4.9	6.6	5.0	5.1	7.2	7.3	6.0	6.1	6.9	6.7	6.9	6.9	6.2	6.5
O_2 Cap., vol. %	15.9	15.9	12.0	12.0	18.3	18.3	17.8	17.8	20.1	20.0	14.9	14.9	16.5	16.5
Art., % sat.	91	91	91	91	91	92	93	95	91	91	94	94	92	92
Art. CO_2, vol. %	48.7	45.2	40.1	39.4	44.4	44.0	47.1	42.7	43.1	42.5	45.3	44.5	44.8	43.1
Art. pH	7.43	7.43	7.43	7.40	7.40	7.40	7.40	7.41	7.41	7.45	7.39	7.39	7.41	7.42
Art. pCO_2, mm. Hg	41	37	32	30	38	37	41	36	38	34	39	38	38.2	35.3
Pulse	76	71	94	100	79	80	68	60	75	75	82	79	79	78
MABP, mm. Hg	102	114	87	93	105	106	113	134	94	96	100	92	100	106
CBF, ml./100 Gm./min.	49	41	46	38	56	51	45	50	44	45	31	31	45	43
CMR O_2, ml./100 Gm./min.	2.4	2.7	2.3	1.9	4.0	3.7	2.7	3.1	3.0	3.0	2.1	2.2	2.8	2.8
CVR	2.1	2.8	1.9	2.45	1.9	2.1	2.5	2.7	2.1	2.1	3.1	3.0	2.1	2.4
CMR gl., mg./100 Gm./min.	3.3	1.02	3.7	2.4	6.7	8.2	5.0	3.4	5.7	5.0	1.8	3.4	4.4	3.9

TABLE III

Glucose, lactate and pyruvate values—high spinal anesthesia

Patient	Art. gluc. mg. % C	Art. gluc. mg. % E	Ven. gluc. mg. % C	Ven. gluc. mg. % E	A-V gluc. mg. % C	A-V gluc. mg. % E	Patient	Art. lact. mg. % C	Art. lact. mg. % E	A-V-lact. mg. % C	A-V-lact. mg. % E	Art. pyruv. mg. % C	Art. pyruv. mg. % E	A-V pyruv. mg. % C	A-V pyruv. mg. % E
F. D.	86	89	67	79	17	10	S. K.	3.6	4.7	−0.7	−0.4	1.03	1.25	−0.12	−0.18
J. R.	91	96	85	90	6.0	6.0	A. B.	4.6	9.4	+1.6	+1.8	1.20	1.54	−0.23	−0.03
B. M.	85	93	82	87	3.0	6.0	G. S.	4.0	8.0	−3.5	−1.6	1.55	1.80	+0.24	−0.11
C. T.	85	84	77	77	8	7	M. B.	5.2	6.4	−2.3	−0.5	1.10	1.30	−0.13	
M. L.	66	71	60	61	6	10	D. S.	4.3	4.7	−0.9	−0.4	1.86	2.78	−0.16	−0.15
S. K.	68	68	59	62	9	6	M. S.					1.08	1.30	+0.12	−0.04
M. B.	99	97	87	96	13	1.0	M. C.*	12.2	13.7			2.02	2.29		
A. G.	96	108	83	100	13	8	M. K.*	14.3	21.6						
M. S.	82	82	66	67	16	15	A. G.*	6.8	8.3			1.36	1.71		
Mean	84.2	87.8	74	79.9	10.1	7.7	Mean	6.9	9.6†	−1.8	−0.26	1.40	1.75†	−0.13	−0.05
							Double Controls								
W. O.	76	75	70	73	6	2	W. O.	5.3	5.0	−0.44	+0.30	1.00	1.10	−0.23	−0.17
D. M.	76	80	67	74	8	6	J. C.	7.3	5.7	−0.06	−0.06	1.60	1.40	−0.23	−0.06
J. C.	114	118	102	102	12	16	B. H.*	4.8	4.8			2.42	2.07		
A. B.	69	69	59	62	10	7	E. T.*	4.7	4.7			1.25	1.26		
V. P.	68	66	55	55	13	11	J. W.*	4.8	5.7			1.19	1.20		
H. R.	79	82	73	71	6	11	R. C.*	8.0	7.6			1.68	1.62		
							P. D.*	7.7	7.4			2.03	1.87		
Mean	80	82	71	73	9.3	8.9	Mean	6.1	5.8			1.60	1.50		

* Taken from studies of Hackel, Sancetta, and Kleinerman (4).
† Signifies a statistically significant difference when compared with the variation in double control series ($p = <0.01$).

The cerebral glucose consumption

The mean CMR gl showed no significant change in the normotensive high spinal group, varying from 4.2 mg. per 100 Gm. per minute in the prespinal study to 3.2 mg. per 100 Gm. per minute in the spinal period. In the double control group the mean values decreased from 4.4 mg. per 100 Gm. per minute in the first study to 3.9 mg. per 100 Gm. per minute in the second, again a change which was not significant. The values in the hypertensive patients during high spinal anesthesia showed no consistent change. The mean change in CMR gl in the normotensives during high spinal anesthesia was not significantly different from the mean change observed in double control studies.

Blood gases

In normotensive patients the mean changes in arterial oxygen content, jugular vein oxygen content, arterial-jugular venous oxygen difference, oxygen capacity and arterial oxygen saturation during high spinal anesthesia were not of statistical significance when compared to the prespinal values. This was also true for the mean changes in arterial CO_2 content, arterial pH and arterial pCO_2 in the normotensives. The small changes in oxygen and carbon dioxide concentration and in arterial pH likewise were not significant when compared to the variations observed in the double control group. In the hypertensive group there were more distinct and consistent decreases in the mean arterial oxygen content, jugular vein oxygen content, and oxygen capacity, and a notable increase in the arterial-jugular vein oxygen difference. It is of interest that the decrease in mean oxygen capacity in the hypertensives during high spinal anesthesia paralleled that of the mean arterial oxygen content so that no change in mean arterial oxygen saturation occurred. The mean arterial CO_2 content and arterial pCO_2 showed small but consistent decreases during high spinal anesthesia as compared to prespinal values. Although the small number of hypertensives studied precluded a statistical analysis, there was a suggestive difference between the normotensive and hypertensive groups in the response of oxygen values and carbon dioxide contents and pressures to spinal anesthesia.

Carbohydrate metabolites

The mean arterial glucose and venous glucose levels showed slight increases during high spinal anesthesia and similar but smaller increases during the double control studies. The mean arterial-jugular vein glucose difference showed a slight decrease in the normotensive spinal group and a similar but smaller decrease in the double control group. The hypertensives behaved similarly in this regard. These changes in the spinal group when analyzed against the double control changes were not statistically significant.

In a small series of five determinations there appeared to be a slight increase in the mean arterial lactate level during high spinal anesthesia. This was not seen in two double control studies. A slight increase was also consistently present in the mean arterial pyruvate level in six high spinal studies, but was seen in only one of two arterial pyruvate studies done in the control series. There were no consistent changes in the mean arterial-jugular vein lactate and pyruvate differences of the high spinal and the double control groups. However, the mean arterial-jugular venous lactate difference, although negative, was less negative in four of five observations during high spinal anesthesia. These results are summarized in Table III.

DISCUSSION

These studies indicate the cerebral vessels in normotensive persons can dilate sufficiently to maintain an entirely adequate cerebral circulation despite the pronounced decrease in mean arterial blood pressure caused by high spinal anesthesia. On the basis of our limited studies in hypertensives, these persons do not appear to have the same capacity for cerebral vascular compensation as do the normotensives. This is in agreement with the results obtained by Kety, King, Horvath, Jeffers, and Hafkenschiel (5) in hypertensives.

The MABP in the normotensive group fell approximately 32 per cent from the control value while the hypertensives decreased to 50 per cent of the control value during the spinal anesthetic studies. During differential spinal block the mean blood pressure drop was 26 per cent in Kety's study. This difference undoubtedly is a reflection of the higher anesthetic level and greater anesthetic

dosage in the present study (above T_4 with evidence of vascular dilatation in the upper extremities). The greatest absolute drop in MABP was obtained in the four hypertensive patients, but the absolute level of the MABP during spinal anesthesia in these hypertensives was no different than the arterial pressure level in the normotensives during spinal anesthesia. The greater decrease in arterial pressure in the four hypertensives as compared to the normotensives is consonant with the known greater reactivity of these patients to spinal anesthesia. Kety and his co-workers pointed to the correlation between the change in mean arterial blood pressure and the change in internal jugular oxygen content as evidence of inadequate cerebral circulatory homeostasis at the greater blood pressure drops. Our limited data in hypertensives corroborate this observation and suggest an inability of the hypertensive patients to obtain complete cerebral circulatory compensation during the severe hypotensive episodes. The arterial-jugular venous oxygen content in the hypertensives showed a consistent increase during the high spinal study, a finding which would be in keeping with a decreased cerebral blood flow if cerebral oxygen consumption is to be maintained.

It is of interest that the arterial lactate and arterial pyruvate levels showed a consistent increase during spinal anesthesia as compared to the prespinal values. Because our observations were numerically inadequate to evaluate these findings, we have combined the present data with those previously reported by our group in another facet of this study (4). This compilation of material is reasonable since all determinations were performed by the same laboratory, using the same technique and methods. Table III indicates that there was a constant and statistically significant increase in the arterial lactate and pyruvate levels during high spinal anesthesia, when compared to the changes observed in the double control group. The arterial glucose and cerebral arteriovenous differences of glucose, lactate, and pyruvate showed no such consistent change.

The mechanism of this increase in arterial lactate and pyruvate is not clear. It seems possible that anoxia of one or several organ systems may be responsible for the lactate and pyruvate changes. Of the systems studied, the heart (4), the splanchnic bed (3), and the kidneys (17) suffer a decreased blood flow incident to the hypotension of high spinal anesthesia. Evidence has been presented that the hepatosplanchnic oxygen consumption remains unaltered despite a decreased blood flow. The splanchnic bed appears to compensate for the decreased flow by an increased arteriovenous oxygen extraction. The heart, in spite of a decreased oxygen consumption, has a markedly decreased work load and the myocardial oxygen extraction coefficient is not increased. This does not preclude the possible development of an unrecognized oxygen debt. The effects of spinal anesthesia on the renal circulation are more difficult to assess. Recent studies (17) of the effects of high spinal anesthesia in pregnant women point to a decrease in renal plasma flow and an associated increase in renal vascular resistance. No studies of renal oxygen consumption were made. However, earlier studies of spinal anesthesia (18, 19), in which the anesthetic level was lower, showed no consistent change in renal blood flow. The critical difference between these results appear to be the effect of the spinal anesthetic on the blood pressure. In the former study, hypotension was produced, whereas the blood pressure level in the latter studies was not affected. It is reasonable to assume that when spinal anesthesia is associated with hypotension, as was the case in our studies, a decrease in renal blood flow can occur, and renal anoxemia may occur. This organ may conceivably be the source of the increased lactate and pyruvate levels during the high spinal studies. It is, of course, possible that temporary ischemia of the liver and splanchnic bed, not discernible by short term physiologic studies, may also contribute.

The mechanism of the decreased cerebrovascular resistance during high spinal anesthesia remains obscure. It is unlikely that there was a direct effect on the cerebral vessels, since the anesthetic level was never above T_1 and previous studies (20) have shown that bilateral block of the stellate ganglion does not produce a decreased cerebrovascular resistance in normotensive or hypertensive patients. Another mechanism which might decrease the cerebrovascular resistance during high spinal anesthesia is a decrease in the pCO_2 or pH of the cerebral tissues. The jugular-venous blood reflects these changes in the cerebral tissues. We have not gathered sufficient data on

jugular-venous pH and pCO_2 to implicate this mechanism, but it seems unlikely that metabolic local changes in the cerebral tissues of sufficient degree to alter pH or pCO_2 could occur in the absence of any change in cerebral oxygen uptake, cerebral blood flow, or jugular venous oxygen content. It is possible that local baroreceptors (21) in the carotid arteries and sinus may produce a reflex vasodilation of the cerebral vessels in response to hypotension.

While these results indicate that there is complete circulatory compensation in the brain during the hypotension of high spinal anesthesia in normotensive persons, it must be emphasized that this response is dependent upon local ability to vasodilate and decrease vascular resistance. Foci with vascular sclerosis or other disease may not be able to compensate completely, and may therefore be liable to ischemia. Since the technique employed gives values only for total cerebral circulation and metabolism, small foci of brain receiving a diminished blood flow may not be detected.

CONCLUSIONS

1. The effects of the hypotension induced by high spinal anesthesia on the cerebral circulation and metabolism of 13 subjects have been studied. Nine of these were normotensive and four were hypertensive. A series of six double control studies served for comparison. In the normotensive group given high spinal anesthesia the MABP fell from a prespinal level of 93 to 63 mm. Hg, but the CBF did not decrease significantly, and the CMR O_2 was unchanged. The CVR decreased significantly and was responsible for the maintenance of the cerebral blood flow in the face of the 32 per cent decrease in MABP.

In a small group of hypertensive patients, the MABP fell to 50 per cent of the prespinal value during high spinal anesthesia. Suggestive decreases were found in CBF and CVR but the CMR O_2 did not appear to change in this group.

2. There were no significant changes in the blood gases and pH in the normotensive group during high spinal anesthesia. The arterial oxygen content, jugular venous oxygen content and oxygen capacity appeared to decrease and the arterial jugular vein oxygen difference to increase in hypertensives during high spinal anesthesia. The arterial pCO_2 and arterial CO_2 contents showed consistent decreases during spinal anesthesia in the hypertensive patients.

3. The arterial blood lactate and pyruvate were significantly elevated during high spinal anesthesia associated with hypotension. It is suggested that renal or splanchnic ischemia may be responsible for these effects.

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REFERENCES

1. Lynn, R. B., Sancetta, S. M., Simeone, F. A., and Scott, R. W. Observations on the circulation in high spinal anesthesia. Surgery 1952, **32**, 195.
2. Sancetta, S. M., Lynn, R. B., Simeone, F. A., and Scott, R. W. Studies of hemodynamic changes in humans following induction of low and high spinal anesthesia. I. General considerations of the problem. Circulation 1952, **6**, 559.
3. Mueller, R. P., Lynn, R. B., and Sancetta, S. M. Studies of hemodynamic changes in humans following induction of low and high spinal anesthesia. II. The changes in splanchnic blood flow, oxygen extraction and consumption, and splanchnic vascular resistance in humans not undergoing surgery. Circulation 1952, **6**, 894.
4. Hackel, D. B., Sancetta, S. M., and Kleinerman, J. Effect of hypotension due to spinal anesthesia on coronary blood flow and myocardial metabolism. Circulation 1956, **13**, 92.
5. Kety, S. S., King, B. D., Horvath, S. M., Jeffers, W. A., and Hafkenschiel, J. H. The effects of an acute reduction in blood pressure by means of differential spinal sympathetic block on the cerebral circulation of hypertensive patients. J. clin. Invest. 1950, **29**, 402.
6. Bessman, A. N., Alman, R. W., and Fazekas, J. F. Effect of acute hypotension on cerebral hemodynamics and metabolism of elderly patients. Arch. intern. Med. 1952, **89**, 893.
7. Hafkenschiel, J. H., Crumpton, C. W., Moyer, J. H., and Jeffers, W. A. The effects of dihydroergocornine on the cerebral circulation of patients with essential hypertension. J. clin. Invest. 1950, **29**, 408.
8. Simeone, F. A., Craney, J. J., Grass, A. M., Linton, R. R., and Lynn, R. B. An oscillographic plethysmograph using a new type of transducer. Science 1952, **116**, 355.
9. Hickam, J. B., and Frayser, R. Spectrophotometric

determination of blood oxygen. J. biol. Chem. 1949, **180**, 457.

10. Peters, J. P., and Van Slyke, D. D. Quantitative Clinical Chemistry, Volume II, Methods. Baltimore, Williams and Wilkins, 1932.
11. Rosenthal, T. B. The effect of temperature on the pH of blood and plasma *in vitro*. J. biol. Chem. 1948, **173**, 25.
12. Singer, R. B., and Hastings, A. B. An improved clinical method for the estimation of disturbances of the acid-base balance of human blood. Medicine 1948, **27**, 223.
13. Somogyi, M. Determination of blood sugar. J. biol. Chem. 1945, **160**, 69.
14. Barker, S. B., and Summerson, W. H. The colorimetric determination of lactic acid in biological material. J. biol. Chem. 1941, **138**, 535.
15. Friedemann, T. E., and Haugen, G. E. Pyruvic acid. II. The determination of keto acids in blood and urine. J. biol. Chem. 1943, **147**, 415.
16. Kety, S. S., and Schmidt, C. F. The nitrous oxide method for the quantitative determination of cerebral blood flow in man: Theory, procedure and normal values. J. clin. Invest. 1948, **27**, 476.
17. Assali, N. S., Kaplan, S. A., Foman, S. J., Douglass, R. A., and Tada, Y. The effect of high spinal anesthesia on the renal hemodynamics and the excretion of electrolytes during osmotic diuresis in the hydropenic normal pregnant woman. J. clin. Invest. 1951, **30**, 916.
18. Smith, H. W., Rovenstine, E. A., Goldring, W., Chasis, H., and Ranges, H. A. The effects of spinal anesthesia on the circulation in normal, unoperated man with reference to autonomy of arterioles, and especially those of the renal circulation. J. clin. Invest. 1939, **18**, 319.
19. Mokotoff, R., and Ross, G. The effect of spinal anesthesia on the renal ischemia in congestive heart failure. J. clin. Invest. 1948, **27**, 335.
20. Harmel, M. H., Hafkenschiel, J. H., Austin, G. M., Crumpton, C. W., and Kety, S. S. The effect of bilateral stellate ganglion block on the cerebral circulation in normotensive and hypertensive patients. J. clin. Invest. 1949, **28**, 415.
21. Green, J. H. Further baroceptor areas associated with the common carotid artery in the cat. J. Physiol. 1954, **123**, 41 p.

THE EFFECT OF ABRUPT CHANGES IN PLASMA CALCIUM CONCENTRATIONS ON RENAL FUNCTION AND ELECTROLYTE EXCRETION IN MAN AND MONKEY[1,2]

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It is well known that patients with hypercalcemia of diverse etiologies may exhibit marked polyuria (1–5). The increased rate of urine formation is often associated with considerable losses of urinary solute and sometimes results in severe depletion of extracellular salt and water stores (6). This urinary wastage has been attributed to slowly developing pathological changes in the renal tubules produced by prolonged hypercalciuria and nephrocalcinosis. It is possible, however, that the renal response may in part be conditioned by a direct and immediate physiological effect of the hypercalcemia and/or hypercalciuria. The studies of Wolf and Ball in which calcium infusions in the dog provoked a prompt increase in the rate of sodium excretion (7) suggest that such is the case. To test this hypothesis further, experiments were devised in man and monkey to evaluate the immediate effects of changing filtered calcium loads upon discrete renal functions and upon the rate of excretion of electrolytes.

MATERIALS AND METHODS

An increase in the filtered load of calcium was achieved by a sustained calcium infusion or by the single intravenous injection of calcium in the form of gluconate or lactate. In monkey and man the infusions were administered in the form of calcium gluconate or lactate at the rate of 0.07 mg. of calcium per minute per Kg. for a period of 60 to 90 minutes. The calcium salts were dissolved in hypotonic saline solutions (0.45 per cent). The infusion was administered at a constant rate of 1 ml. per minute in man and 0.1 ml. per minute in monkey and contained proper quantities of inulin and PAH for the measurement of renal clearances. Comparable solutions free of calcium were administered at the same rate before and after the calcium infusion. Three to four 20 minute control periods were obtained prior to the calcium infusion, during which inulin and PAH clearances and the control rates of sodium, chloride, potassium, calcium, phosphorus and ammonium (pH and bicarbonate in some experiments) excretion were determined. Similar measurements were made in four 20 minute periods during the calcium infusion, and for two to three comparable periods after the calcium infusion was discontinued. Standard catheter and air flush techniques were used for bladder emptying and clearance determinations. During the control period at least two and generally three heparinized blood samples were obtained for the determination of plasma calcium, phosphorus, sodium, potassium, chloride, bicarbonate, inulin and PAH concentrations. Similar measurements were repeated at 15 minute intervals during the calcium infusion and after its discontinuation. The calcium infusion studies were performed in five resting normal adults in a fasting state and in three normal fasting cynomolgus monkeys. Similar measurements were made in man before and after a single intravenous injection of 10 ml., 20 ml., and 40 ml. of 10 per cent calcium gluconate solution, respectively. These single injection experiments were performed in seven normal fasting male subjects.

After the typical response to a standard rapid injection of 20 ml. of calcium gluconate was evaluated, similar loads were administered to eight patients in whom the urine had been either alkalinized or acidified with appropriate prior therapy. In the latter subjects sodium bicarbonate or ammonium chloride solutions of 150 mEq. per liter were administered at the rate of 2 ml. per minute for 90 minutes before and for a similar period after the injection of the calcium load. In these subjects, measurements similar to those mentioned above were made during the acidifying or alkalinizing infusion both before and after the administration of the calcium load.

The plasma calcium concentration was reduced in three normal patients by the infusion of a chelating solution. Sodium Versenate® (sodium salt of ethylenediaminetetraacetic acid, Riker Laboratories, Inc.) was infused at the rate of 15 mg. per minute for 40 minutes. Measurements identical to those listed above were repeated before, during and for 90 minutes after the chelate

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infusion. In addition, free and total (digested) calcium concentration in the urine was measured during and after the Versenate® infusion.

The chemical methods are, for the most part, identical to those previously used and reported from this laboratory (8, 9). In addition, phosphorus was analyzed by the method of Fiske and Subbarow (10) and calcium by the technique of Kramer and Tisdall (11).

RESULTS

Calcium infusions in man (Table I)

The plasma calcium concentration rose promptly during the calcium infusion, remained elevated throughout the infusion and tended to fall toward normal in the postinfusion period (Figure 1, Table I). The plasma phosphorus rose consistently but much more slowly, reaching its highest levels after the discontinuation of the infusion in four of the five subjects (Table I). The peak increases in plasma calcium and phosphorus concentration averaged 6.0 mg. per cent (2.7 to 12.0) and 1.5 mg. per cent (0.9 to 1.9), respectively. No changes were noted in plasma sodium, potassium, chloride, bicarbonate concentrations or in the plasma pH.

The rate of calcium excretion began to increase promptly after the onset of the calcium infusion and reached a maximum toward the end of the infusion period (Figure 1, Table I), with the increment averaging 56 μEq. per minute (44 to 62). Phosphorus excretion also rose after the onset of the calcium infusion. This peak increase averaged 21 μM per minute and tended to occur somewhat later than the maximal increase in the rate of calcium excretion.

The calcium infusions induced a prompt increase in the rate of salt excretion (Figure 1, Table I). This increase was noted in the first infusion period, reached a peak toward the end of the calcium infusion, and persisted to a lesser extent during the postinfusion period. The maximum increase in the rate of sodium excretion averaged 700 μEq. per minute (375 to 1,086 μEq. per minute) or more than four times the control values. The simultaneous peak increase in the rate of chloride excretion averaged 520 μEq. per minute (190 to 870 μEq. per minute) or greater than two times the control values. Coincident with the peak increase in salt excretion, there occurred a moderate increase in the rate of urine

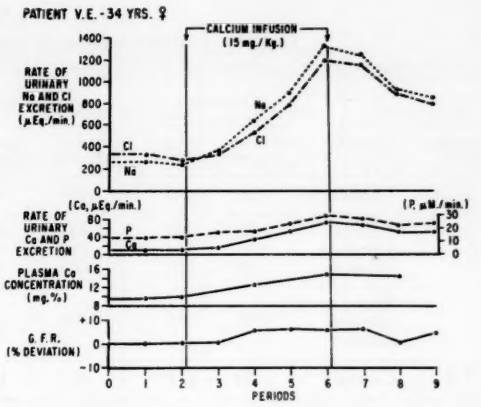

FIG. 1. THE EFFECT OF A CALCIUM INFUSION ON RENAL FUNCTION, ELECTROLYTE EXCRETION, CALCIUM AND PHOSPHORUS PLASMA CONCENTRATIONS IN MAN

flow averaging 3 ml. per minute. The increases in the rate of calcium, salt and water excretion tended to coincide. The rate of potassium excretion did not change consistently during the calcium infusion (Table I), tending to increase in two experiments, fall in two and remain unchanged in one. No consistent changes in the rate of urinary excretion of ammonia, bicarbonate, or in urinary pH were noted during or after the calcium infusions in those experiments in which these indices were measured.

Inulin clearance measurements showed no detectable changes in three of the human subjects and a small increase in the two remaining subjects (Table I—S. E. and V. E.). PAH clearances showed an increase in four of the five subjects (Table I).

Calcium infusions in monkey (Table II)

The data obtained from calcium infusions in three monkeys were similar to those obtained in man (Figure 2, Table II). The changes in plasma calcium and phosphorus concentrations corresponded to those seen in man with the rise in phosphorus tending to occur more slowly and reaching its peak later. The maximum increases in calcium and phosphorus concentrations averaged 2.6 and 0.9 mg. per cent, respectively. The peak increase in the rate of calcium excretion averaged 14 μEq. per minute and tended to coincide

TABLE I

The effects of calcium infusion on renal function and electrolyte excretion in man

Subject	Periods	Clearances		Urine electrolyte excretion					Urine volume	Plasma concentration	
		Inulin *ml./min.*	PAH *ml./min.*	Sodium *μEq./min.*	Chloride *μEq./min.*	Potassium *μEq./min.*	Calcium *μEq./min.*	Phosphorus *μM/min.*	*ml./min.*	Calcium *mg. %*	Phosphorus *mg. %*
R. R.	Control*	103	553	293	300	76	3.3	12.1	3.2	9.4	3.3
	Calcium gluconate infusion	114	688	434	387	82	8.2	20.1	6.9	12.6	4.1
		109	654	609	449	34	36.7	21.3	3.9		
		100	650	666	490	34	47.1	23.0	1.2	13.7	
	After calcium infusion	103	592	582	471	28	44.8	28.9	1.3	13.4	4.8
S. E.	Control*	74	412	217	149	53	4.3	2.3	1.4	9.3	2.1
	Calcium gluconate infusion	81	470	357	316	73	14.8	5.6	4.5	21.4	2.3
		93	546	1,211	848	92	62.3	13.4	5.0	16.4	
	After calcium infusion	82	437	803	534	102	47.6	10.1	3.3	15.0	3.7
		71	428	617	425	74	31.6	7.0	2.9		
V. E.†	Control*	95	321	284	332	78	6.2	11.7	1.8	9.3	3.7
	Calcium gluconate infusion	96	364	387	370	62	12.6	15.5	1.8	12.2	4.3
		108	413	675	582	47	31.9	17.6	3.2		
		108	410	934	822	49	48.4	21.8	4.6	14.6	4.7
		108	487	1,370	1,240	64	70.2	27.0	8.5		
	After calcium infusion	109	448	1,291	1,198	67	67.5	25.6	7.1		
		97	405	950	923	50	47.5	20.6	4.5	14.0	5.2
		106	433	875	814	68	45.5	21.3	4.6		
R. M.	Control*	126	590	130	228	140	10.4	4.9	5.7	9.2	2.6
	Calcium gluconate infusion	128	624	263	415	130	22.8	4.5	5.4	10.0	2.6
		122	608	536	473	116	40.4	8.4	4.0	12.4	
		123	640	731	612	121	71.7	13.7	5.0	15.0	3.2
	After calcium infusion	127	610	578	477	103	67.5	14.5	2.8		3.5
N. L.	Control*	121	632	239	229	98	18.1	0.9	2.7	9.9	2.1
	Calcium gluconate infusion	127	676	414	388	127	36.8	1.6	9.4	11.2	2.2
		125	608	705	641	97	65.2	5.2	6.9		
		124	555	761	691	77	74.7	9.0	5.3	12.6	4.0
	After calcium infusion	123	531	658	590	62	69.8	11.8	4.1	11.2	2.9
		120	670	448	418	50	57.8	11.5	2.3		

* Controls represent the average of three 20 minute periods. Subsequent periods varied between 20 and 25 minutes in length.

† In this patient 0.15 mg. of calcium per Kg. per minute was administered.

with the increases in the rate of sodium and chloride excretion. The maximal increments in the rate of sodium and chloride excretion averaged 22 and 14 μEq. per minute or about six times the control values, respectively. In contrast to man, a consistent increase in the rate of potassium excretion averaging 7 μEq. per minute occurred simultaneously with the maximum rate of salt excretion. No changes in inulin clearance were noted during or after the calcium infusion.

TABLE II

The effects of calcium infusion on renal function and electrolyte excretion in the monkey

Monkey	Periods	Clearances		Electrolyte excretion					Plasma concentration	
		Inulin *ml./min.*	PAH *ml./min.*	Sodium *μEq./min.*	Chloride *μEq./min.*	Potassium *μEq./min.*	Calcium *μEq./min.*	Ammonia *mg./min.*	Calcium *mg. %*	Phosphorus *mg. %*
I	Control*	10.6		0.4	2.4	6.3	6.6		12.6	2.4
	Calcium gluconate infusion	10.0		0.6	3.4	5.0	7.4			
		11.4		2.0	6.5	11.7	13.7		14.0	2.7
		9.1		3.4	10.1	11.8	15.9			
		9.4		1.2	5.6	6.5	16.6		15.6	2.7
		8.6		2.3	8.7	9.4	17.0			
	After calcium infusion	9.7		1.2	4.5	5.1	10.2			
		11.4		0.9	2.7	2.2	13.7		11.8	3.5
		7.5		0.9	2.4	1.5	5.6			
II	Control*	14.8		6.1	12.3	6.3	10.2	7.0	11.4	2.5
	Calcium gluconate infusion	12.7		7.3	13.7	6.5	12.1	6.5	14.2	2.6
		13.7		23.0	37.9	12.2	21.0	9.9		
		13.0		21.0	36.4	9.4	20.3	8.4	15.6	3.5
		12.5		22.6	35.5	8.0	15.5	6.5		
	After calcium infusion	11.4		10.3	22.1	6.5	17.1	5.9		
		11.2		8.0	14.3	5.2	12.8	5.0	11.1	3.2
		10.6		7.2	14.5	4.8	10.1	4.8		
III	Control*	14.5	79.7	1.9	3.4	5.7	5.1	6.1	11.6	4.2
	Calcium gluconate infusion	15.1	86.7	6.0	15.4	6.7	14.7	9.4	12.4	4.6
		15.7	90.7	14.1	37.8	10.2	26.2	14.3		
		13.9	81.3	13.1	34.0	9.0	24.7	9.8	12.6	5.8
		15.0	87.8	22.7	37.0	13.4	29.6	11.2		
	After calcium infusion	14.3	90.3	5.1	28.8	4.1	20.6	4.2		
		14.1	89.1	0.9	7.4	1.6	15.7	8.5	9.4	5.4
		12.6	83.0	1.1	3.3	3.3	7.8	7.9		

* Controls represent the average of three 20 minute periods. Each subsequent period varied between 20 and 25 minutes in length.

Single intravenous administration of calcium in man (Tables III and IV)

The rapid intravenous administration of 10, 20, and 40 ml. of 10 per cent calcium gluconate solution in man likewise produced an increase in the rate of calcium and salt excretion with the precise response proportionate to the quantity injected (Table III). Following the 20 ml. load, the rate of calcium and phosphorus excretion increased an average of 17 μEq. and 13 μM per minute, respectively. Coincidentally, the increment in the rate of sodium and chloride excretion averaged 183 and 148 μEq. per minute, or 200 and 110 per cent greater than the control rates, respectively. A small transient increase in potassium excretion was noted but no changes in urinary pH or bicarbonate concentration were noted. The changes following the injection of a 40 ml. calcium load were qualitatively similar but greater, whereas those following the injection of 10 ml. of calcium gluconate solution were considerably smaller (Table III). No changes in glomerular filtration rate or renal plasma flow were detected in those subjects in which it was measured following the single intravenous calcium injections (Table III—M. V., S. S., and J. M.)

In the experiments in which 20 ml. of calcium gluconate was administered intravenously to subjects being infused with bicarbonate or ammonium chloride, no definite difference in the response to the calcium load was noted from that observed in the unprepared patients (Table IV). The increase in the rate of calcium, sodium, and chloride excretion approximated that observed in patients

TABLE III

The effects of a single calcium gluconate injection on renal function and electrolyte excretion in man

Subject	Periods	Clearances		Urine electrolyte excretion							Urine		Plasma concentration	
		Inulin	PAH	Sodium	Chloride	Potassium	Calcium	Phosphorus	CO_2	Ammonia	Volume	pH	Calcium	Phosphorus
		ml./min.	*ml./min.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*μM/min.*	*vol. %*	*mg./min.*	*ml./min.*		*mg. %*	*mg. %*
M. V.	Control*	84	449	242	248	70	4.7	10.9		60.0	1.4		9.1	3.3
				10 ml. of calcium gluconate injected										
		93	587	351	350	80	7.4	15.4		58.7	4.4		9.0	3.3
	After calcium gluconate injection	86	427	326	323	47	12.3	13.0		60.3	5.0			
		89	466	365	335	48	14.0	12.3		56.0	4.7		11.2	
		87	468	365	330	49	14.3	12.3		58.5	4.8			
		79	418	316	312	43	13.4	10.6		55.6	3.3		9.6	3.8
		83	435	318	308	46	12.2	11.1		60.0	3.5			
L. G.	Control*			120	135	29	2.5	13.5	6.0		8.2	5.93		
				20 ml. of calcium gluconate injected										
				328	280	43	12.1	39.9	6.3		17.3	6.09	12.6	3.5
	After calcium gluconate injection			324	285	45	17.4	37.2	6.3		12.4	6.06		
				201	188	34	14.5	32.9	5.5		17.0	6.04	11.8	3.8
				167	165	31	14.4	28.3	5.3		12.5	5.95		
				136	142	28	13.5	23.0	5.3		13.8	5.95	10.8	3.8
C. J.	Control*			107	165	133	5.2	21.9	15.6		9.8	6.60	10.8	3.9
				20 ml. of calcium gluconate injected										
				330	361	218	30.2	32.9	17.1		16.3	6.65		
	After calcium gluconate injection			239	253	120	24.4	23.3	15.5		6.3	6.49		
				200	215	119	22.1	22.6	16.2		14.8	6.60	11.4	3.7
				117	117	93	15.4	17.0	13.4		13.4	6.52		
				147	134	101	20.1	19.3	12.5		17.5	6.57		
				118	124	77	15.1	15.6	12.5		13.8	6.59	12.2	3.6
C. J.	Control*			91	140	83	10.0	3.9	12.9		6.5	6.68	10.2	2.6
				20 ml. of calcium gluconate injected										
				299	347	157	14.1	7.7	15.3		20.0	6.74		
	After calcium gluconate injection			300	334	130	34.6	10.8	14.4		21.6	6.70		
				196	219	105	25.6	8.9	12.1		18.3	6.63		
				169	214	122	26.1	8.5	11.5		19.3	6.62		
				85	114	71	16.1	4.6	12.1		11.9	6.64	11.2	2.8
				140	192	122	29.6	8.2	12.1		21.1	6.60		
				106	119	86	21.5	6.5	11.3		14.8	6.54	10.8	3.1

* Controls represent the average of three 20 minute periods. Subsequent periods varied between 20 and 25 minutes in length.

TABLE III—*Continued*

Subject	Periods	Clearances		Urine electrolyte excretion							Urine		Plasma concentration	
		Inulin	PAH	Sodium	Chloride	Potassium	Calcium	Phosphorus	CO_2	Ammonia	Volume	pH	Calcium	Phosphorus
		ml./min.	*ml./min.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*μM/min.*	*vol. %*	*mg./min.*	*ml./min.*		*mg. %*	*mg. %*
S. A.	Control*			132	146	55	7.5	0.9	26.5		9.6	6.57	11.0	1.8
				20 ml. of calcium gluconate injected										
	After calcium gluconate injection			220	174	63	10.2	0.9	28.5		17.0	6.60		
				225	183	53	15.2	1.0	26.8		17.7	6.55		
				207	181	52	13.6	1.1	25.6		18.2	6.48	11.2	2.3
				185	178	65	11.9	0.9	23.3		17.0	6.32		
				153	160	57	8.9	1.1	23.3		14.8	6.34		
				161	168	56	9.1	1.2	23.3		14.0	6.39		
				183	190	61	11.0	1.7	23.6		14.7	6.46	13.0	2.7
S. S.	Control*	129	542	267	269	128	11.0	14.7		69.4	5.0		9.6	3.6
				40 ml. of calcium gluconate injected										
	After calcium gluconate injection	130	532	517	422	135	28.3	27.1		85.0	9.7		11.0	3.8
		138	478	667	520	92	47.8	32.9		85.4	11.8		11.0	4.3
		118	427	467	398	64	30.4	24.5		61.9	6.3		10.8	4.1
		126	462	422	374	65	28.2	24.1		68.1	7.0		10.2	4.2
J. M.	Control*	125	688	243	240	59	11.5	11.3		81.0	3.7		9.6	3.2
				40 ml. of calcium gluconate injected										
	After calcium gluconate injection	131	794	577	564	93	35.8	16.3		93.9	10.5		12.0	3.5
		128	686	764	737	83	54.1	24.2		66.5	6.0		10.8	3.7
		132		638	592	71	43.4				6.6			
		127	674	397	384	48	39.1	27.0		95.8	10.6		10.4	4.2

whose urine had not been previously acidified or alkalinized. At the time that the calcium load was administered, the urine pH's averaged 6.01, 6.52, and 7.33 in the acidified group, the untreated, and the alkalinized group, respectively. No change in the plasma pH was detected during the acid or the alkaline infusions.

Versenate® infusion in man (Table V)

In the chelate experiments, as the plasma calcium concentration fell, the rate of sodium and chloride excretion likewise decreased (Figure 3, Table V). Plasma calcium concentration began to fall toward the end of the chelating infusion and continued to fall thereafter. Total calcium excretion, free plus chelated (digested), rose promptly after the onset of the infusion but the rate of excretion of free calcium fell toward the end of the Versenate® infusion. Coincidentally, there occurred a fall in the rate of sodium and chloride excretion. These falls in electrolyte excretion averaged 177 and 144 μEq. per minute or 45 and 47 per cent of the control values for sodium and chloride, respectively. Total phosphorus excretion tended to rise slowly after the onset of the Versenate® infusion reaching its highest levels after the cessation of the infusion. No changes in glomerular filtration rate or renal plasma flow were noted throughout these chelate experiments.

DISCUSSION

These data indicate that an increase or decrease in the plasma calcium concentration promptly produces a similar change in the rate of salt excretion

TABLE IV

The effects on electrolyte excretion of a single calcium injection in patients receiving an acid or alkaline infusion

Subject	Periods	Urine electrolyte excretion					Urine volume	Urine pH	Plasma pH	Plasma concentration	
		Sodium	Chloride	Potassium	Calcium	Phosphorus				Calcium	Phosphorus
(Infused with NH_4Cl)		*μEq./min.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*μM/min.*	*ml./min.*			*mg. %*	*mg. %*
P. L.	Control*	85	118	61	8.4	8.3	4.5	6.45		10.4	1.9
		20 ml. of calcium gluconate injected									
	After calcium gluconate	216	208	60	34.7	16.0	12.6	6.32			
		222	239	46	21.6	19.5	12.7	6.18			
		110	123	24	12.8	10.8	7.5	6.11			
		145	181	33	19.6	14.6	11.5	6.07			
A. A.	Control*	151	156	36	13.5	10.2	0.8	6.21	7.23	10.1	1.4
		20 ml. of calcium gluconate injected									
	After calcium gluconate	231	208	29	14.8	8.0	5.6	6.18	7.32	11.0	1.8
		154	149	11	15.1	6.7	1.2	5.51			
		205	225	14	20.8	7.5	1.2	5.48	7.34	10.6	2.0
		220	246	14	21.6	6.7	1.2	5.47			
R. B.	Control*	27	57	20	6.2	4.7	0.5	5.50	7.36	10.0	
		20 ml. of calcium gluconate injected									
	After calcium gluconate	143	180	38	20.9	6.6	1.3	5.46	7.38	12.7	
		96	117	25	23.7	5.9	0.7	5.02			
		116	168	21	31.1	7.9	0.9	5.09	7.39	11.0	
		159	225	23	28.1	6.7	1.5	5.09			
D. M.	Control*	155	242	123	6.7	4.0	9.7	6.82	7.34	11.1	1.7
		20 ml. of calcium gluconate injected									
	After calcium gluconate	287	335	126	19.8	6.6	7.6	6.70	7.36	13.8	
		246	304	100	17.6	6.6	3.9	6.60			
		213	282	88	15.1	7.9	2.9	6.44	7.36		
		228	337	113	15.1	9.9	5.7	6.32			

* Controls represent the average of three 20 minute periods. Subsequent periods varied between 20 and 25 minutes in length.

TABLE IV—*Continued*

Subject	Periods	Urine electrolyte excretion: Sodium	Chloride	Potassium	Calcium	Phosphorus	Urine volume	Urine pH	Plasma pH	Plasma concentration: Calcium	Phosphorus
(*Infused with $NaHCO_3$*)		*μEq./min.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*μM/min.*	*ml./min.*			*mg. %*	*mg. %*
V. V.	Control*	404	189	106	2.8	23.8	6.7	7.74		9.5	2.8
		20 ml. of calcium gluconate injected									
		491	245	70	4.2	25.7	11.8	7.16		10.6	2.9
	After	477	264	38	6.2	21.2	15.5	6.90			
	calcium	447	240	44	7.1	18.9	16.7	6.81		9.9	3.1
	gluconate	396	204	42	7.8	14.3	18.2	7.07			
		384	222	31	9.0	8.0	9.0	7.39			
G. I.	Control*	309	142	176	4.6	23.3	4.9	7.66		10.0	2.4
		20 ml. of calcium gluconate injected									
		473	225	157	15.1	21.0	4.5	7.63		10.4	2.5
	After	430	188	95	19.2	23.1	10.4	7.29			
	calcium	199	72	63	8.7	18.9	10.8	7.12			
	gluconate	205	60	78	8.0	10.2	13.4	7.12			
		266	77	87	10.5	10.3	12.3	7.29			
S. L.	Control*	121	43	181	7.5	36.3	6.7	7.20		10.2	2.2
		20 ml. of calcium gluconate injected									
		319	100	272	27.0	39.3	21.8	7.17		10.3	2.2
	After	214	46	79	20.3	19.6	10.5	7.15			
	calcium	229	50	94	28.7	28.4	14.2	7.00			
	gluconate	160	24	48	20.9	23.9	10.7	7.03			
		199	28	58	23.7	22.1	11.8	7.09			
P. L.	Control*	360	116	198	5.3	8.8	6.6	7.70		9.3	2.1
		20 ml. of calcium gluconate injected									
		565	172	145	23.5	31.8	9.3	7.36			
	After	299	87	53	14.4	26.2	4.3	7.36			
	calcium	188	46	44	9.6	21.3	6.6	7.01			
	gluconate	148	44	69	11.2	25.9	9.7	7.03			
		236	39	64	9.5	23.7	7.3	7.23			

in man and monkey. The mechanism by which this change in the rate of electrolyte excretion occurs is not certain. It is possible that a change in the rate of electrolyte excretion could be explained by a similar change in glomerular filtration rate. Indeed, in two of the five calcium infusion studies in man an increase in filtration rate was observed coincident with the peak increase in salt excretion. However, in the remaining experiments in man and monkey, no measurable changes in filtration rate were apparent. Furthermore, in those experiments where a slight increase in filtration rate did occur, the markedly elevated rate of salt excretion persisted after the filtration rate had returned to control levels (Figure 1, Table I). The changes in salt excretion are compared with the changes in filtration rate in Figure 4, but no consistent relation is apparent. In the chelate infusion experiments the falls in electrolyte excretion were not associated with a measurable fall in filtration rate. These data, therefore, show no consistent trend to suggest that the changes in salt excretion are mediated through changes in glomerular filtration rate. Instead, they imply that a change in plasma calcium concentration and in the rate of calcium excretion effects an immediate and opposite change in the rate of tubular reabsorption of sodium and chloride. Figure 5 suggests that this altered tubular function correlates best with the change in the rate of calcium excretion.

The contention that the salt diuresis is caused by the osmotic load imposed by the calcium does not appear tenable. The salt diuresis produced

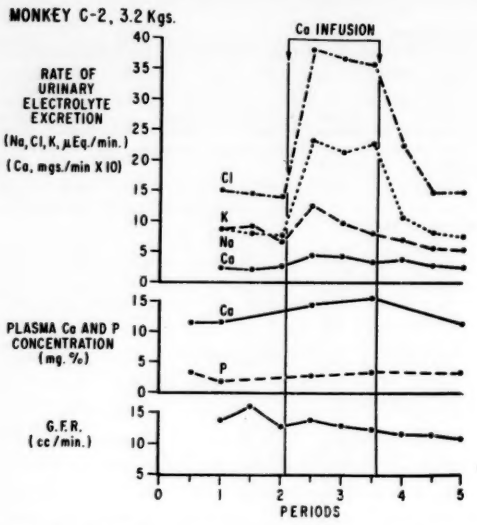

FIG. 2. THE EFFECT OF A CALCIUM INFUSION ON RENAL FUNCTION, ELECTROLYTE EXCRETION, CALCIUM AND PHOSPHORUS PLASMA CONCENTRATIONS IN MONKEY

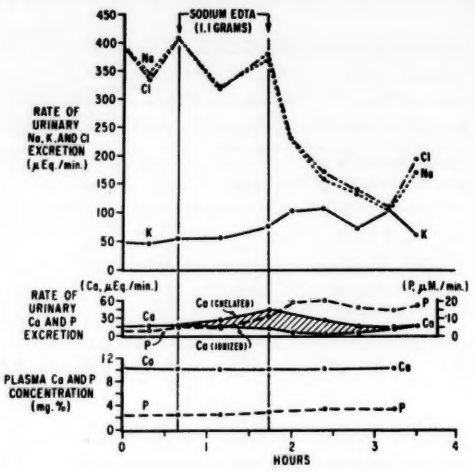

FIG. 3. THE EFFECT OF A CHELATING INFUSION ON PLASMA CALCIUM CONCENTRATION, FREE CALCIUM EXCRETION, RENAL FUNCTION AND ELECTROLYTE EXCRETION IN MAN

by the calcium infusions in man averaged almost twenty times in total solute that due to the calcium *per se*. Similarly, after the chelate infusions, the fall in excretion of salt far exceeded in total solute the coincident decrease in free calcium excretion.

The site in the tubular lumen or in the tubular cells affected by a change in the rate of calcium excretion remains unknown. It has been demonstrated in isolated flounder tubules that changing calcium concentration affects the rate of phenol red transport (12). Of interest is the fact that considerable changes in the urinary pH by acid or alkaline infusions did not appreciably alter the saluresis of a standardized calcium load (20 ml. calcium gluconate intravenously).

The consistent and prompt increase in the rate of phosphorus excretion and the slower rise in plasma phosphorus concentration produced by the administration of calcium deserve some comment. It has been repeatedly demonstrated that a calcium infusion in normal man will reduce the overall rate of phosphorus excretion in the subsequent 24 hour period (13, 14). However, it has also been noted that comparable calcium loads in hypoparathyroid subjects, or in normal subjects in whom the rate of phosphorus excretion is measured during the infusion and shortly thereafter, produce an immediate increase in phosphorus excretion (13–15). This latter observation appears in substantial agreement with those reported here. The apparent discrepancy suggests that the calcium infusion evokes a prompt increase in the rate of phosphorus excretion prior to its slower and opposite effect in reducing the rate of excretion. This latter change has been attributed to the gradual suppression of parathyroid function with a consequent increase in the rate of phosphorus reabsorption by the renal tubules (13, 14).

The cause of the more immediate increase in phosphorus excretion is not established. It is conceivable that the calcium infusion promptly stimulated parathyroid function with a consequent reduction in tubular reabsorption of phosphorus. However, this alternate explanation is not consistent with the large body of evidence which argues that a calcium infusion suppresses parathyroid function (13, 14, 16, 17). It is likely that, in part at least, this increased rate of excretion simply reflects the rising plasma phosphorus concentrations produced by the administration of calcium. However, the change in excretory rate seems more impressive and appears to precede the slower rise in plasma phosphorus concentration. It is tempting to attribute this phosphate diuresis in large part to the immediate alteration in tubular function effected by the calcium administration. In this view, the reduced capacity for the reabsorption of sodium chloride and phosphorus may

TABLE V

The effects of a chelating infusion on renal function and electrolyte excretion in man

Subject	Periods	Clearances		Urine electrolyte excretion						Urine volume	Plasma concentration	
							Calcium					
		Inulin	PAH	Sodium	Chloride	Potassium	Free	Digested	Phosphorus		Calcium	Phosphorus
		ml./min.	*ml./min.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*μEq./min.*		*μM/min.*	*ml./min.*	*mg. %*	*mg. %*
S. C.	Control*	124	555	346	353	127	7.2	7.2	4.1	5.3	9.2	2.9
	EDTA (76 ml.) infusion	134	670	335	331	131	6.6	31.5	5.2	7.2	9.1	3.4
		124	576	298	258	144	5.3	60.6	8.9	4.9	8.6	3.5
	After EDTA infusion	135	473	219	225	140	2.4	58.6	11.8	6.9	8.8	3.3
		114	466	185	191	116	0.6	43.0	14.5	5.1		
W. R.	Control*	169	649	372	380	136	15.1	15.1	37.3	8.5	10.7	5.1
	EDTA (60 ml.) infusion	156	677	339	331	124	15.3	21.4	42.2	9.1	10.4	4.9
		161	667	292	258	118	14.5	22.8	49.3	9.3	9.8	4.9
	After EDTA infusion	158	610	273	233	112	10.9	22.4	48.5	7.8	9.8	4.7
		152	596	247	228	93	8.2	23.0	37.1	5.6		
		170	605	253	228	84	8.5	15.9	48.3	3.3	10.6	4.7
D. B.	Control*	126	825	379	343	49	16.6	16.6	3.8	3.9	10.9	2.4
	EDTA (110 ml.) infusion	128	802	321	319	55	15.1	22.2	4.1	2.6	10.0	2.4
		142	900	369	382	75	12.0	44.7	10.7	2.8	10.0	2.7
	After EDTA infusion	136	898	231	231	102	5.2	36.4	18.6	8.0	10.2	3.2
		122	879	157	162	106	2.9	26.5	20.3	12.1		
		122	740	133	139	71	5.0	15.7	16.0	3.4		

* Controls represent the average of three 20 minute periods. Subsequent periods varied between 20 and 25 minutes in length.

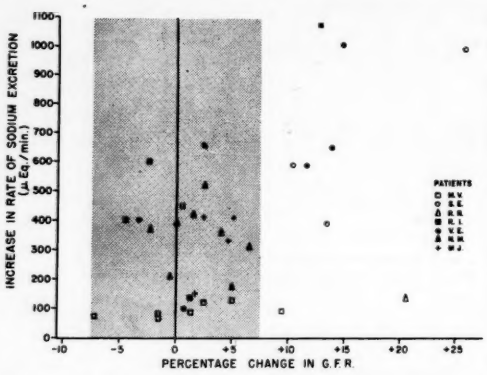

FIG. 4. CHANGES IN SODIUM EXCRETION PLOTTED AGAINST CHANGES IN GLOMERULAR FILTRATION RATE

Shaded areas represent changes of ± 7.5 per cent in filtration rate—changes which are within the experimental error.

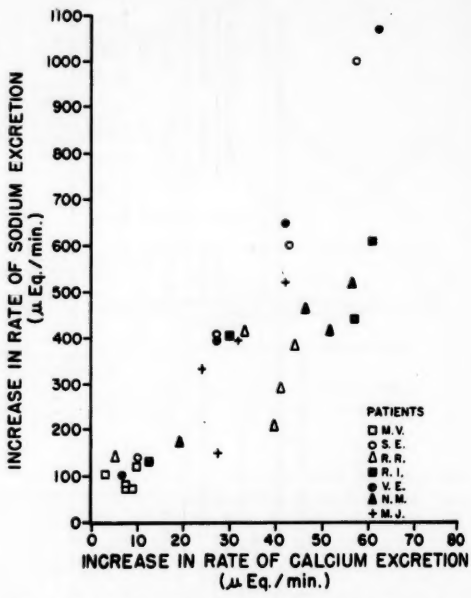

FIG. 5. CHANGES IN SODIUM EXCRETION IN THE INFUSION STUDIES IN MAN PLOTTED AGAINST CHANGES IN THE RATE OF EXCRETION OF CALCIUM

all represent the direct effect of a sudden increase in filtered calcium load.

The slow rise in plasma phosphorus concentration noted toward the end of the calcium infusion and thereafter agrees with similar observations reported by others (17, 18). This finding has been attributed to the rapid release of phosphorus from some source such as bone or tissue cells. These data are in accord with such a hypothesis but do not help to delineate the site from which the phosphorus stores were transferred nor the mechanism by which hypercalcemia induces such a transfer.

The slight tendency towards an increase in phosphorus excretion toward the end and after the chelating infusion may best be explained by an increased secretion of parathyroid hormone. An increased rate of hormone secretion would be expected to result from a falling plasma calcium level (19, 20) induced by the increased urinary loss of calcium in the chelated form.

The difference between the consistent increase in potassium excretion in the monkey and the inconsistent changes in man after a comparable calcium load may reflect the basic dietary habits of both species. The monkey maintained on a predominantly fruit diet responds like any subject on a sodium free regimen. If an anion load is imposed by a calcium induced chloruresis, potassium is excreted because less sodium is available (21). An alternate hypothesis to explain this difference would place the calcium effect directly on sodium reabsorption at a site proximal to the potassium secreting segment.

Whether the salt diuresis induced by hypercalcemia might ultimately prove of therapeutic value in the edematous subject remains to be seen. As a separate form of therapy or combined with other known diuretic agents, such calcium loads might prove therapeutically feasible.

SUMMARY

1. Calcium infusions in man and monkey at the rate of 0.1 mg. calcium per minute per kilogram produce a prompt increase in the rate of sodium, chloride, and water excretion.

2. Single intravenous administration of 10, 20, and 40 ml. of 10 per cent calcium gluconate solution in man produces a comparable increase in the rate of salt and water excretion in proportion to the dose administered.

3. The calcium infusions or single intravenous injections evoke a prompt increase in the rate of phosphorus excretion.

4. The effects of a single intravenous injection of calcium are not appreciably modified by the prior acidification or alkalinization of the urine.

5. A reduction in the plasma calcium concentration by the administration of a chelating agent causes a fall in the rate of salt excretion.

REFERENCES

1. Mandl, F. Klinisches und experimentelles zur frage der lokalisierten und generalisierten ostitis fibrosa. Arch. klin. Chir. 1926, **143**, 245.
2. Gutman, A. B., Swenson, P. C., and Parsons, W. B. The differential diagnosis of hyperparathyroidism. J. Amer. med. Ass. 1934, **103**, 87.
3. Lightwood, R. Idiopathic hypercalcaemia in infants with failure to thrive. Arch. Dis. Childh. 1952, **27**, 302.
4. Dietrick, J. E., Whedon, G. D., and Shorr, E. Effect of immobilization upon various metabolic and physiological functions of normal man. Amer. J. Med. 1948, **4**, 3.
5. Gribetz, D., Sweet, A. Y., Becker, A. M., Levitt, M. F., and Hodes, H. L. The use of anabolic steroids to reduce hypercalcemia and hypercalciuria caused by immobilization in paralytic poliomyelitis. In press.
6. Schelling, D. H. Parathyroids in Health and Disease. St. Louis, C. V. Mosby, 1935.
7. Wolf, A. V., and Ball, S. M. Effect of intravenous calcium salts on renal excretion in the dog. Amer. J. Physiol. 1949, **158**, 205.
8. Levitt, M. F., Turner, L. B., Sweet, A. Y., and Pandiri, D. The response of bone, connective tissue, and muscle to acute acidosis. J. clin. Invest. 1956, **35**, 98.
9. Levitt, M. F., Turner, L. B., and Sweet, A. Y. The effect of experimental venous obstruction on salt and water distribution and excretion in man. J. clin. Invest. 1952, **31**, 885.
10. Fiske, C. H., and Subbarow, Y. The colorimetric determination of phosphorus. J. biol. Chem. 1925, **66**, 375.
11. Kramer, B., and Tisdall, F. F. The direct quanitative determination of sodium, potassium, calcium and magnesium in small amounts of blood. J. biol. Chem. 1921, **48**, 223.
12. Puck, T. T., Wasserman, K., and Fishman, A. P. Some effects of inorganic ions on the active transport of phenol red by isolated kidney tubules of the flounder. J. cell. comp. Physiol. 1952, **40**, 73.
13. Howard, J. E., Hopkins, T. R., and Connor, T. B. On certain physiological responses to intravenous injection of calcium salts into normal, hyperparathyroid and hypoparathyroid persons. J. clin. Endocr. 1953, **13**, 1.
14. Hiatt, H. H., and Thompson, D. D. Some effects of intravenously administered calcium on inorganic phosphorus metabolism. J. clin. Invest. 1957, **36**, 573.
15. Jackson, W. P. U., Hoffenberg, R., Linder, G. C., and Irwin, L. Syndrome of steatorrhea, pseudohypoparathyroidism and amenorrhea. J. clin. Endocr. 1956, **16**, 1043.
16. Albright, F., and Reifenstein, E. C., Jr. The Parathyroid Glands and Metabolic Bone Disease. Baltimore, Williams and Wilkins, 1948.
17. Baylor, C. H., Van Alstine, H. E., Keutmann, E. H., and Bassett, S. H. The fate of intravenously administered calcium. Effect on urinary calcium and phosphorus, fecal calcium and calcium-phosphorus balance. J. clin. Invest. 1950, **29**, 1167.
18. Salvesen, H. A., Hastings, A. B., and McIntosh, J. F. The effect of the administration of calcium salts on the inorganic composition of the blood. J. biol. Chem. 1924, **60**, 327.
19. Albright, F. The parathyroids—physiology and therapeutics. J. Amer. med. Ass. 1941, **117**, 527.
20. Patt, H. M., and Luckhardt, A. B. Relation of a low blood calcium to parathyroid secretion. Endocrinology 1942, **31**, 384.
21. Schwartz, W. B., Jenson, R. L., and Relman, A. S. The disposition of acid administered to sodium-depleted subjects: The renal response and the role of the whole body buffers. J. clin. Invest. 1954, **33**, 587.

DISPLACEMENT OF NITROGEN FROM NORMAL HUMAN LUNGS DURING OXYGEN BREATHING[1]

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Abnormalities of intrapulmonary distribution and mixing of inspired gases occur in diseases of the lungs, particularly in pulmonary emphysema. Interest in this problem began in 1864, and has been pursued with many different methods (1). However, uncertainty and even confusion still exists not only in the definition but also in the quantitative measurement of intrapulmonary distribution and mixing. One means of investigating the problem is the measurement of nitrogen displacement from the lung as it occurs during oxygen breathing, reported by Cournand and his associates (2–4). As an index of impaired distribution and mixing of the inspired gas with alveolar gas, they proposed an end-expired nitrogen concentration exceeding 2.5 per cent, delivered by a forced expiration at the end of a seven minute period of quiet oxygen-breathing (3). With such a technique only two points in the course of the nitrogen dilution were known—the initial alveolar nitrogen concentration while breathing air, and the final nitrogen concentration produced by the terminal forced expiration. Subsequently, a mathematical expression was presented (4) for ideal intrapulmonary mixing of oxygen and nitrogen, enabling predictions of the intrapulmonary nitrogen concentration at the end of any given number of breaths of oxygen. Divergence was found even in normal persons between the actual course of nitrogen dilution and the predicted course.

With the help of continuous rapid automatic gas analyzers such as the nitrogen meter (5) and the mass spectrometer (6), later investigators could follow the breath-by-breath dilution of nitrogen during the breathing of oxygen. After graphical analysis of the displacement curves they proposed interesting concepts of well-ventilated and poorly-ventilated lung compartments, and of rates of gas mixing in these spaces (6–8). Simpler criteria were proposed by others (9–13), in particular "turnover indices" which related functional residual capacity to the total ventilation required to dilute the intrapulmonary nitrogen to a given endpoint (10, 12, 13).

Previous workers have studied relatively few normal human subjects. The purposes of the present paper are to present data on the quantitative aspects of nitrogen displacement from the lungs of fifty normal persons, as a basis of comparison for the findings in patients with pulmonary disease, and to suggest certain clinically valuable criteria for the evaluation of intrapulmonary gas mixing. From this investigation normal nitrogen clearance characteristics have been determined, and the physiological factors which influence clearance have been evaluated.

MATERIAL AND METHODS

Fifty medical students free from pulmonary disease were selected for study, on the basis of a health questionnaire and review of Student Health Service records, which included recent chest radiographs and physical examinations.

The open-circuit method of Cournand, Baldwin, Darling, and Richards (3) for measurement of functional residual capacity was modified to include continuous monitoring of the expired nitrogen by a nitrogen meter with a recording milliammeter.[2] The combined accuracy of our nitrogen meter and recording system varied from + 0.1 per cent to − 0.2 per cent (mean, − 0.05 per cent in 51 trials) in the range of 0 to 20 per cent nitrogen, and amounted to ± 0.5 per cent in the 20 per cent to 80 per cent nitrogen range, as determined by comparison with manometric gas analysis (14). Minimum readability in the 0 to 20 per cent range was 0.1 per cent nitrogen; in the 20 per cent to 80 per cent range it was 0.5 per cent

[1] This work was supported in part by a grant from the Los Angeles County Tuberculosis and Health Association.

[2] The nitrogen meter was manufactured by the Waters Company, Rochester, Minnesota. The recorder was manufactured by the Esterline-Angus Company, Indianapolis, Indiana.

nitrogen. The nitrogen meter was calibrated before and after each run with a series of known mixtures of nitrogen and oxygen, bubbled through water.

The subjects, in a nonbasal state, rested for 10 to 15 minutes before being tested. All tests were made in the supine position. Air was breathed by mouthpiece through a four-way respiratory valve, two arms of which were connected to a balloon-in-bottle system containing air, one arm to a Douglas bag containing oxygen, and one arm to a 120 liter Tissot spirometer. A spirometer record of the tidal volume changes in the balloon was obtained during air-breathing. When it was apparent that breathing was even and that the expiratory base line was steady, the subject was switched from air to cylinder oxygen exactly at the end of a normal expiration. With this arrangement, a switch made too soon or too late could be corrected in the calculation of functional residual capacity. For the next seven minutes the subject inspired oxygen from the Douglas bag and expired into the Tissot spirometer. The volume of each expiration was recorded on the Tissot kymograph, which was started simultaneously with the nitrogen meter recorder at the instant oxygen-breathing began. The nitrogen meter sampled continuously from a point just distal to an expiratory flap valve near the mouthpiece. Thus, a breath-by-breath analysis of the end-expired nitrogen concentration and expiratory volume was made. At the end of the seventh minute a forced expiration was made and a sample of gas was drawn into a syringe from a point adjacent to the nitrogen meter sampling connection, following which the subject was switched back to air-breathing. The final concentrations of nitrogen and carbon dioxide in the Tissot spirometer were measured by manometric (14) and volumetric (15) methods, as well as by the nitrogen meter. The final forced expiratory sample was analyzed for carbon dioxide. The peak end-expired nitrogen concentration was considered "alveolar" ($F_{A_{N_2}}$), as was the carbon dioxide concentration in the final forced expiration ($F_{A_{CO_2}}$).

The Tissot kymograph record was used to calculate tidal volumes, respiratory rates, and minute ventilation (all volumes in this report are corrected to body temperature and pressure, saturated with water vapor). Functional residual capacity (FRC) was calculated according to the previously described method (3), correcting for diffusion of tissue nitrogen into the alveoli (16). Suitable corrections were made for valve dead space and for deviations from the true expiratory base line when the subject was switched to oxygen. Alveolar ventilation was computed according to a derivation from the Bohr formula (17): $V_A/V_E = F_{E_{CO_2}}/F_{A_{CO_2}}$, where V_A is alveolar ventilation, V_E is total expired ventilation, $F_{A_{CO_2}}$ is alveolar carbon dioxide (*vide supra*), and $F_{E_{CO_2}}$ is the volume fraction of carbon dioxide in the mixed expired (spirometer) gas (18). From the record of the nitrogen meter and from the spirometer kymograph record of collected expired gas, a graph was prepared plotting the logarithm of 100 ($F_{A_{N_2}} - F_{I_{N_2}}$), which is the difference between end-expired nitrogen and inspired nitrogen (usually 0.5 per cent to 0.6 per cent in the cylinder oxygen), expressed as per cent, on the ordinate against total expired volume (V_E) or against total alveolar volume (V_A) on the abscissa. The points on the graph represent nitrogen-volume values for frequently selected breaths during the entire period of oxygen-breathing. This type of graph, the so-called washout curve, is shown in Figure 1.

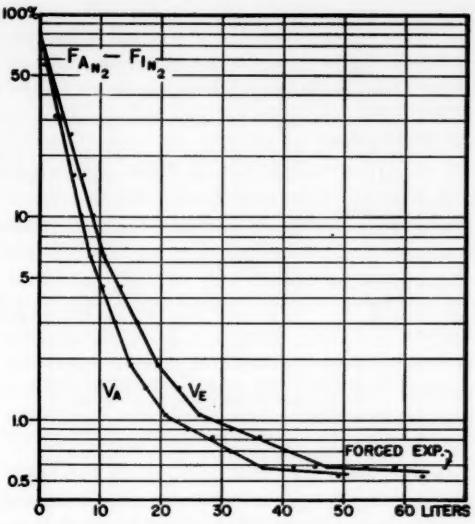

FIG. 1. GRAPH OF DECLINE IN EXPIRED NITROGEN CONCENTRATION (END-TIDAL) AS A FUNCTION OF TOTAL EXPIRED GAS VOLUME (V_E) AND OF ALVEOLAR COMPONENT OF TOTAL EXPIRED GAS VOLUME (V_A), DURING OXYGEN BREATHING FOR SEVEN MINUTES

$F_{A_{N_2}} - F_{I_{N_2}}$ = per cent expired N_2 minus per cent inspired N_2.

RESULTS

The physical characteristics of the subjects are given in Table I. Also tabulated are the relevant features of their respiration which might affect the rate of nitrogen displacement. The subjects have been divided into three groups. Group I is composed of 33 men whose resting expiratory base line before the change from air-breathing to oxygen-breathing was constant enough to permit confidence in the computed FRC. Their mean FRC was 2.29 liters ± 0.51 (S. D.). Group II consists of 12 men whose FRC measurements could not be considered as exact as in Group I because of difficulty in reaching a steady expiratory level in the period of time allowed before the switch to oxygen. They are not included in the evaluation of

TABLE I

Characteristics of fifty normal young adults

Group	Age	Height	Weight	B.S.A.*	FRC*	f*	V_T*	$\dot{V}_E$*	V_A/V_E*
	yrs.	*in.*	*lbs.*	M^2	*liters*		*ml.*	*liters/min./M^2*	
I. 33 males									
Mean	24.1	70.7	162.7	1.92	2.295	11.0	803	4.13	0.66
S. D.	2.3	2.7	19.3	0.14	0.512	4.2	281	0.79	0.08
II. 12 males									
Mean	23.1	71.1	172.3	1.98	2.543	9.7	956	4.32	0.70
S. D.	1.6	2.4	28.3	0.17	0.373	3.1	401	0.88	0.08
III. 5 females									
Mean	26.2	65.3	128.6	1.64	1.742	13.5	500	4.06	0.62
S. D.	7.7	2.9	18.8	0.15	0.302	3.3	60	0.72	0.06

* B.S.A. = Body surface area, in square meters.
FRC = Functional residual capacity, in liters.
f = Number of respirations per minute.
V_T = Tidal volume, in ml.
$\dot{V}_E$ = Minute volume of expired gas, in liters per square meter B.S.A.
V_A/V_E = Alveolar fraction of total expired gas.

the initial or steep part of the nitrogen washout curve, where size of FRC exerts its greatest influence (*vide infra*). However, their washout behavior in the latter part of the seven minute period is regarded as satisfactory for inclusion with the other subjects because, despite variation of the initial FRC, intrapulmonary mixing should be complete, or almost so, at the end of seven minutes in these normal young individuals. Review of the data showed this assumption to be correct. The data for five females are listed separately in Table I as Group III. Their mean FRC was 1.74 liters ± 0.30 (S. D.).

The values for, and the variability of, FRC among our subjects confirm the findings of others (19–21) who have measured pulmonary volume compartments in normals (Table II). In agreement with these investigators (19–22), no close correlation was found between FRC and various parameters of body size, including the cube of the height as suggested by Bateman (23).

The ratio V_A/V_E, which relates total ventilation to alveolar ventilation, depends on $F_{A_{CO_2}}$ in a single forced expiration and has the inherent errors of dead space determination (24), yet offers the advantages of utility. The mean values in Groups I, II, and III are 0.66, 0.70, and 0.62, respectively, and are close to the generally accepted normal value of 0.70 (25).

The nitrogen clearance curves are presented for simplicity in two phases—the initial rapid drop of end-expired nitrogen as a function of summated expired ventilation (V_E) or of summated alveolar ventilation (V_A), and the subsequent slow decline. Previous workers (6, 7) have attempted, by plotting log $F_{E_{N_2}}$ against time or number of breaths

TABLE II

Comparative values for functional residual capacity in literature and in present study

Investigators	No. of subjects	Sex	Age	Posture	Mean FRC	Variability of FRC
			yrs.		*liters*	
Kaltreider (19)	50	M	18–30	Supine	2.18	0.50 (S. D.)
	50	F	18–30	Supine	1.82	0.39 (S. D.)
Needham (20)	27	M	20–30	Seated	3.21	1.9–4.3
	18	F	20–30	Seated	2.34	1.7–3.5
Whitfield (21)	41	M	10–70	Supine	2.459	0.591 (S. D.)
	16	F	10–70	Supine	1.856	0.458 (S. D.)
Present study	33	M	21–31	Supine	2.295	0.512 (S. D.)
	5	F	21–38	Supine	1.742	0.302 (S. D.)

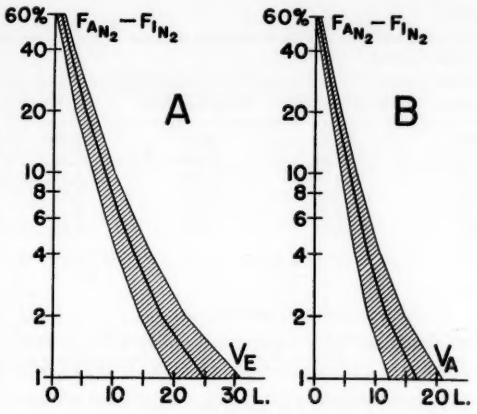

FIG. 2. A—COURSE OF DECLINE OF END-TIDAL NITROGEN CONCENTRATION TO 1 PER CENT AS FUNCTION OF TOTAL EXPIRED GAS VOLUME, DURING OXYGEN BREATHING; B—SAME, RELATING DECLINE IN NITROGEN CONCENTRATION TO ALVEOLAR VENTILATION

Heavy central lines represent mean values for Group I (33 subjects). Cross-hatching represents one standard deviation from the mean. $F_{AN_2} - F_{IN_2}$ = per cent expired N_2 minus per cent inspired N_2.

on semilogarithmic paper, to divide the clearance curves into one or more straight-line segments, and to interpret these segments as representing clearance from separate pulmonary compartments. In the washout curves presented here for normal individuals, where log $(F_{AN_2} - F_{IN_2})$ is plotted against expired volume, no constant number of straight-line segments could be determined. In many instances a single straight line could be drawn through all the nitrogen concentration points for the first few breaths, followed by one or more somewhat less steep straight-line segments. However, the length, slope, and number of these segments varied from one individual to another; hence the attempt to plot straight-line segments to match exactly all observed points was abandoned and instead lines were drawn to fit a majority of the points by inspection. The transition of the initial rapid decline of nitrogen concentration into the later slow decline was also variable from one person to another, but in all subjects took the form of a smooth bend. A convenient end-point for the initial rapid washout phase was arbitrarily chosen as the ventilation point on the curves which coincided with the 1 per cent $(F_{AN_2} - F_{IN_2})$ line. This total observed volume of expired gas is designated V_E (1 per cent), and the calculated total expired alveolar gas to this point is designated V_A (1 per cent).

Figure 2A shows for Group I the mean plot of V_E against $F_{AN_2} - F_{IN_2}$ down to 1 per cent, together with the standard deviation. The mean V_E (1 per cent) is 25.14 liters ± 5.70 (S. D.), with a coefficient of variation of 22.7 per cent. Figure 2B shows the corresponding relationship for V_A (1 per cent). The mean value for V_A (1 per cent) is 16.55 liters ± 4.46 (S. D.), with a coefficient of variation of 27 per cent. These data are tabulated in Table III.

The main physiological factor determining nitrogen clearance from the lung during the oxygen-breathing period should, theoretically, be the lung

TABLE III

Initial or steep part of clearance curve—Group I

$F_{AN_2} - F_{IN_2}$* (%)	V_E* (liters)		V_A* (liters)		V_E/FRC* (liters)		V_A/FRC* (liters)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
60	0.98	0.66	0.55	0.55	0.42	0.19	0.23	0.18
40	2.36	0.84	1.55	0.62	1.04	0.26	0.67	0.18
20	5.24	1.46	3.54	0.98	2.31	0.34	1.53	0.25
10	8.39	1.74	5.62	1.36	3.70	0.45	2.43	0.30
8	9.52	1.93	6.35	1.52	4.19	0.48	2.75	0.33
6	11.00	2.25	7.29	1.77	4.84	0.53	3.15	0.34
4	13.29	2.78	8.72	2.16	5.84	0.59	3.76	0.41
2	18.03	3.74	11.80	2.92	7.93	0.82	5.09	0.55
1	25.14	5.70	16.55	4.46	10.97	1.29	7.08	0.89

* $F_{AN_2} - F_{IN_2}$ = Per cent N_2 in expired gas minus per cent N_2 in inspired gas.
V_E = Total expired gas volume, or sum of tidal volumes.
V_A = Alveolar component of V_E.
FRC = Functional residual capacity.

TABLE IV

Relation of increasing functional residual capacity to ventilation and time required to lower $F_{A_{N_2}} - F_{I_{N_2}}$ to 1 per cent—Group I *

FRC	No.	V_E (1%)†	V_A (1%)†	V_E (1%)/FRC†	V_A (1%)/FRC†	T (1%)†
liters		*liters*	*liters*	*liters*	*liters*	*minutes*
1.400–1.799	4	19.7	11.6	12.290	7.300	2.29
1.800–2.199	12	22.6	13.7	11.179	6.768	2.77
2.200–2.599	8	23.6	16.6	10.055	7.102	3.21
2.600–2.999	4	31.1	21.5	10.929	7.581	3.42
3.000–3.599	4	35.7	23.9	10.954	7.504	4.04

* Mean values given for each subgroup.
† See Tables I and III for terminology.

volume (FRC) to be cleared. The larger the lung volume the greater should be the total ventilation (either expired air volume V_E or alveolar volume V_A) required for clearance, provided the lung is normal and no poorly ventilated spaces exist. It was, therefore, of interest to plot V_E (1 per cent) and V_A (1 per cent) as a function of the FRC. This has been done in Figures 3A and 3B, based on Table IV. A linear regression formula for the relationship between the two has been obtained and is given in the figures. The correlation coefficient for V_E (1 per cent) with FRC is 0.872, and for V_A (1 per cent) with FRC is 0.745, which are high coefficients for lung volume variables.

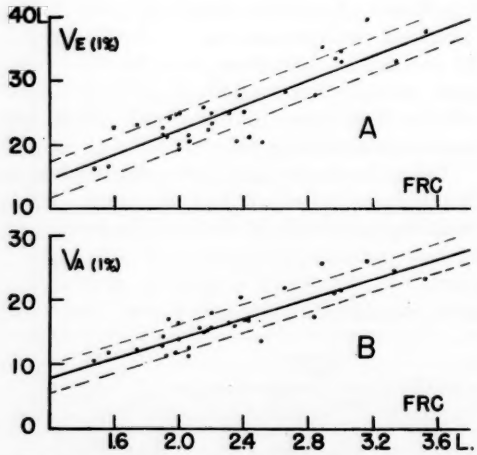

FIG. 3. A—REGRESSION LINE RELATING FUNCTIONAL RESIDUAL CAPACITY (FRC) AND TOTAL EXPIRED GAS VOLUME TO 1 PER CENT NITROGEN; B—REGRESSION LINE RELATING FRC AND ALVEOLAR VENTILATION TO 1 PER CENT NITROGEN

Heavy center lines represent regression formulae: V_E (1 per cent) $= 3.05 + 9.58$ FRC, ± 2.79. V_A (1 per cent) $= -1.41 + 7.71$ FRC, ± 2.27. Dashed lines represent one standard error of estimate from the regression line. Points represent observed values.

Since the size of the FRC influences strongly the ventilation required to dilute the nitrogen to 1 per cent, the ratios V_E (1 per cent)/FRC and V_A (1 per cent)/FRC are proposed for the purpose of minimizing this influence and to allow comparison of individuals with different FRC values. These "turnover ratios" may be expressed as the number of complete replacements of FRC by an equivalent volume of expired gas required to reduce the alveolar nitrogen to 1 per cent, or as the fraction of the ventilation of the total FRC to 1 per cent nitrogen which is required to clear one liter of FRC to this level. The results of using these ratios can be seen in Table IV, and in Figure 4, which show the regression of the ratios on FRC and demonstrate clearly that the ratios change little with increase in FRC. The mean value for Group I for V_E (1 per cent)/FRC is 10.97 ± 1.29 (S. D.), with a coefficient of variation of 11.7 per cent. For V_A (1 per cent)/FRC the mean value is 7.08 ± 0.89 (S. D.), with a coefficient of variation of 12.6 per cent.

The corresponding mean values for the five females at the 1 per cent level are: V_E, 19.52; V_A, 12.22; V_E/FRC, 11.28; and V_A/FRC, 7.04. The close similarity of the turnover ratios to those of the men is to be noted.

In addition to FRC, the efficiency of nitrogen clearance during oxygen-breathing should be influenced by the magnitude of the ratio V_A/V_T (where V_T is tidal volume), since it determines what fraction of the tidal volume penetrates into alveolar regions. A plot of V_E (1 per cent) against V_A/V_T reveals no obviously close rela-

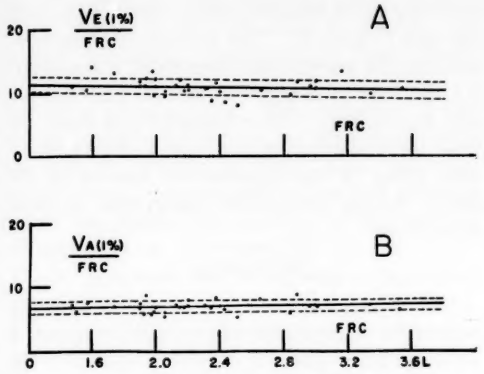

FIG. 4. A—REGRESSION LINE RELATING FUNCTIONAL RESIDUAL CAPACITY (FRC) AND V_E (1 PER CENT)/FRC; B—REGRESSION LINE RELATING FRC AND V_A (1 PER CENT)/FRC

Heavy center lines represent regression formulae: V_E (1 per cent)/FRC = 12.024 − 0.455 FRC, ± 1.224; V_A (1 per cent)/FRC = 6.546 + 0.23 FRC, ± 0.872. Dashed lines represent one standard error of estimate from the regression line. Points represent observed values.

tionship, indicating that in these normal subjects variation in dead space volumes had no apparent effect on the washout curves. This has been noted previously (7).

The mean time required for $F_{A_{N_2}} - F_{I_{N_2}}$ to reach 1 per cent in Group I is 3.06 minutes ± 0.86 (S. D.), with a coefficient of variation of 28.2 per cent (Figure 5). Darling, Cournand, Mansfield, and Richards (2) found that the 1 per cent level was reached in 3.5 to 4 minutes. Luft, Roorbach, and MacQuigg (13) reported that healthy subjects reached "1 per cent, or less" in three to six minutes. The time required to reach 1 per cent depends on ventilation rate, on the size of dead space volume and tidal volume, and the ratio of one to the other, and, of course, on any impairment of intrapulmonary mixing. A hyperventilator will reach the 1 per cent level in a shorter time than a person with lesser ventilation. The advantage of expressing nitrogen clearance as a function of expired volume rather than as a function of time is that it excludes the variable factor of ventilation rate.

Apparently the initial rapid decline in expired nitrogen concentration represents the clearance of well-ventilated areas. This is followed by a flattening out of the curve of nitrogen dilution in

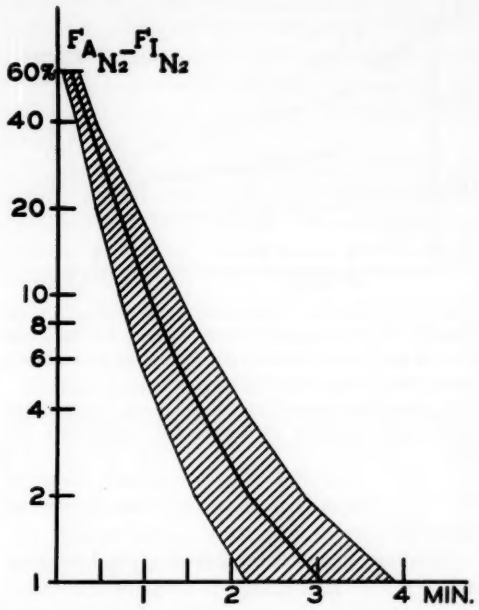

FIG. 5. GROUP I—TIME REQUIRED TO REDUCE END-EXPIRED NITROGEN CONCENTRATION TO 1 PER CENT, DURING OXYGEN BREATHING

Center line represents mean value for 33 subjects. Cross-hatching represents one standard deviation from the mean.

an asymptotic fashion. Roughly, the second half of the seven minute period of oxygen breathing is characterized by a slow decline of end-expired nitrogen levels. The mean values for $F_{A_{N_2}} - F_{I_{N_2}}$ at the end of the fourth minute and at the end of the seventh minute, and the difference between

TABLE V

Behavior of nitrogen decline in last three minutes of washout—Groups I, II, and III combined; 49 subjects

	$F_{A_{N_2}}-F_{I_{N_2}}$* at 4th minute %	$F_{A_{N_2}}-F_{I_{N_2}}$* at 7th minute %	Δ′† %	$F_{A_{N_2}}-F_{I_{N_2}}$* final forced expiration %	Δ″‡ %
Mean	0.72	0.47	0.25	0.53	0.06
S. D.	0.34	0.16	0.29	0.18	0.07

* $F_{A_{N_2}}-F_{I_{N_2}}$ = Per cent N_2 in expired gas minus per cent N_2 in inspired gas.

† Δ′ = Difference between nitrogen concentrations at end of fourth and seventh minutes.

‡ Δ″ = Difference between nitrogen concentrations produced by final forced expiration and the preceding ordinary breath at end of seventh minute.

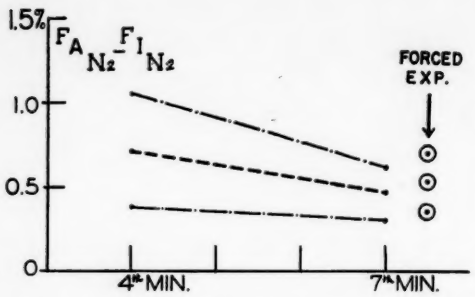

FIG. 6. SCHEMATIC REPRESENTATION OF DECLINE OF NITROGEN CONCENTRATION FROM FOURTH TO SEVENTH MINUTE OF OXYGEN BREATHING, FOLLOWED BY A MAXIMUM EXPIRATION—FORTY-NINE SUBJECTS

Heavy dashed center line represents mean value. Outer dashed lines represent one standard deviation from the mean.

these two values, are shown in Table V and in Figure 6.

The final characteristic of these normal washout curves is the small change in nitrogen concentration produced by a maximal forced expiration at the conclusion of seven minutes of quiet breathing of oxygen. The mean end-expired nitrogen concentration in the terminal forced expiration reached by 49 subjects is 0.53 per cent ± 0.18 (S. D.), expressed at $F_{A_{N_2}} - F_{I_{N_2}}$. The mean increment between this and the last preceding ordinary breath is 0.06 per cent ± 0.07 (S. D.) as shown in Table V. Sixteen out of 49 subjects had no detectable rise; 20 additional subjects had a rise not exceeding 0.1 per cent above the last preceding ordinary breath. Ninety-four per cent of the subjects had no increment greater than 0.2 per cent nitrogen (Figure 7). The greatest increases produced by the forced expirations were, respectively, 0.23 per cent in each of two women, and 0.35 per cent in one man. No adequate explanation for these three aberrant values could be found on review either of the experimental data or of the health records and chest radiographs of these students.

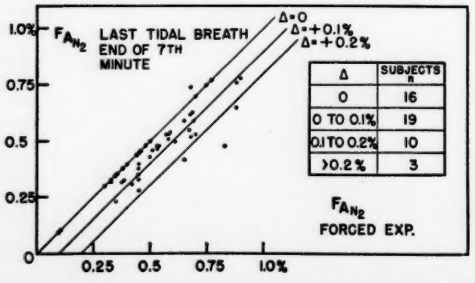

FIG. 7. DIFFERENCE BETWEEN END-TIDAL NITROGEN CONCENTRATION OF LAST TIDAL BREATH AT END OF SEVEN MINUTES OXYGEN BREATHING AND THE NITROGEN CONCENTRATION PRODUCED BY THE TERMINAL FORCED EXPIRATION—FORTY-NINE SUBJECTS

DISCUSSION

It is obvious that the initial or rapidly declining part of the nitrogen displacement curve depicts a well-ventilated space or series of spaces. The later, slowly falling, portions of the curve represent probably a combination of nitrogen displacement from poorly-ventilated alveolar regions and the diffusion of nitrogen from the tissues via the pulmonary circulation into the alveolar gas where the nitrogen tension is lower. How much each of these factors enter into the situation is unknown. The small terminal rise in nitrogen produced by forced expiration would indicate that complete intrapulmonary mixing of oxygen and nitrogen may not occur even after seven minutes in normal persons. This finding confirms the conclusions of Fowler, Cornish, and Kety (*7*, 26).

Calculation of the ventilation in terms of alveolar ventilation involves assumptions not only of constancy of tidal volumes, dead space, and FRC, but also of the validity of a single, forced expiration sample as a true representation of alveolar carbon dioxide. It will be noted in Table II that in the Group I cases the scatter of V_A (1 per cent) leads to a coefficient of variation of 27 per cent. The above mentioned variables may be partially circumvented if analyses are limited to V_E (1 per cent), where the coefficient of variation is 22.7 per cent. Using the ratio V_E (1 per cent)/FRC reduces the scatter still further to a coefficient of variation of 11.7 per cent. Moreover, it allows comparison among individuals with different lung sizes and rates of ventilation. Therefore, the ratio can be considered as a useful index of intrapulmonary mixing. Expressing this ratio as V_A (1 per cent)/FRC does not reduce scatter (coefficient of variation, 12.6 per cent).

A second useful and easily determined index of intrapulmonary mixing is the presence and size of the increment in nitrogen produced by the final

forced expiration. This gives, at a glance, a measure of uniformity of ventilation and indicates the patency of airway communication with poorly ventilated spaces which can be partially emptied by forceful expiration (27). The magnitude of this rise in normal subjects has not been given much attention by previous investigators in connection with prolonged nitrogen washout periods, although Fowler has studied this point by the single-breath nitrogen dilution technique (26).

Luft, Roorbach, and MacQuigg (13), whose experimental procedure resembled that reported here, terminated their nitrogen washout tests when the end-expired nitrogen reached 1.0 per cent or 1.4 per cent, without finishing with a forced expiration. These investigators implied that continued fall in nitrogen below 1 per cent can be attributed to diffusion of the gas from the body tissues. They did not correct, apparently, for inspired nitrogen in the cylinder oxygen. They emphasized the concept of "clearance equivalent," which has been adopted here; namely, the total ventilation to the end-point as the number of replacements of FRC (or the amount of ventilation to clear one volume equivalent to FRC to 1 per cent). This expression eliminates such variable factors as respiratory rate, functional residual capacity, and alveolar ventilation, and reduces total ventilation (cumulative expired volume) to figures which can be used to compare one individual to another. For 25 normal males and 12 normal females, in the seated position, ranging in age from 12 to 64 years, Luft and associates found the mean ventilation to 1 per cent [V_E (1 per cent) in our terminology] to be 44.7 liters in the males and 37.8 liters in the females. Their mean "clearance equivalents" [V_E (1 per cent)/FRC in our terminology] were 13.3 for the males and 13.9 for the females. These values are greater than those in the present report probably because the initial FRC of Luft's subjects was larger as a result of the seated posture, and because of lack of correction for inspired nitrogen.

SUMMARY

1. A method for studying the course of intrapulmonary nitrogen dilution during oxygen breathing by means of a nitrogen meter was incorporated into a well-known procedure for open circuit measurement of functional residual capacity. This method is simple enough to be used clinically.

2. In a group of fifty normal recumbent young adults the course of the nitrogen dilution was followed for seven minutes, and terminated by a forced expiration.

3. Values are given for clearance ventilation to 1 per cent end-expired nitrogen (minus inspired nitrogen), expressed both for total ventilation and alveolar ventilation. These values were found to depend largely upon the size of the functional residual capacity. Clearance equivalent ratios are given which allow comparisons among individuals with different functional residual capacities.

4. The characteristics of the latter half of the washout curves, after the 1 per cent level of nitrogen has been passed, have been investigated with particular emphasis on the usefulness of the final forced expiration.

5. The procedure is a practical method for detecting the presence of uneven alveolar ventilation.

ACKNOWLEDGMENT

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REFERENCES

1. Rauwerda, P. E. Unequal ventilation of different parts of the lung and the determination of cardiac output. Thesis, Groningen, Groningen Univ., 1946.
2. Darling, R. C., Cournand, A., Mansfield, J. S., and Richards, D. W., Jr. Studies on the intrapulmonary mixture of gases. I. Nitrogen elimination from blood and body tissues during high oxygen breathing. J. clin. Invest. 1940, **19**, 591.
3. Cournand, A., Baldwin, E. DeF., Darling, R. C., and Richards, D. W., Jr. Studies on intrapulmonary mixture of gases. IV. The significance of the pulmonary emptying rate and a simplified open circuit measurement of residual air. J. clin. Invest. 1941, **20**, 681.
4. Darling, R. C., Cournand, A., and Richards, D. W., Jr. Studies on intrapulmonary mixture of gases. V. Forms of inadequate ventilation in normal and emphysematous lungs, analyzed by means of breathing pure oxygen. J. clin. Invest. 1944, **23**, 55.
5. Lilly, J. C. Studies on the mixing of gases within the respiratory system with a new type nitrogen meter. Fed. Proc. 1946, **5**, 64.
6. Robertson, J. S., Siri, W. E., and Jones, H. B. Lung ventilation patterns determined by analysis of

nitrogen elimination rates; use of the mass spectrometer as a continuous gas analyzer. J. clin. Invest. 1950, **29**, 577.

7. Fowler, W. S., Cornish, E. R., Jr., and Kety, S. S. Lung function studies. VIII. Analysis of alveolar ventilation by pulmonary N_2 clearance curves. J. clin. Invest. 1952, **31**, 40.
8. Bateman, J. B. Studies of lung volume and intrapulmonary mixing. Nitrogen clearance curves: Apparent respiratory dead space and its significance. J. appl. Physiol. 1950, **3**, 143.
9. Boothby, W. M., Lundin, G., and Helmholz, H. F., Jr. A gaseous nitrogen elimination test to determine pulmonary efficiency. Proc. Soc. exp. Biol. (N. Y.) 1948, **67**, 558.
10. Wolfe, W. A., and Carlson, L. D. Studies of pulmonary capacity and mixing with the N_2 meter. J. clin. Invest. 1950, **29**, 1568.
11. Colldahl, H., and Lundin, G. Ventilatory studies of the lungs in asthma. Acta allerg. (Kbh.) 1952, **5**, 37.
12. Becklake, M. B. New index of intrapulmonary mixture of inspired air. Thorax 1952, **7**, 111.
13. Luft, U. C., Roorbach, E. H., and MacQuigg, R. E. Pulmonary nitrogen clearance as a criterion of ventilatory efficiency. Amer. Rev. Tuberc. 1955, **72**, 465.
14. Peters, J. P., and Van Slyke, D. D. Quantitative Clinical Chemistry, Volume II, Methods. Baltimore, The Williams & Wilkins Co., 1932.
15. Scholander, P. F. Analyzer for accurate estimation of respiratory gases in one-half cubic centimeter samples. J. biol. Chem. 1947, **167**, 235.
16. Cournand, A., Yarmush, I. G., and Riley, R. L. Influence of body size on gaseous nitrogen elimination during high oxygen breathing. Proc. Soc. exp. Biol. (N. Y.) 1941, **48**, 280.
17. Methods in Medical Research, J. H. Comroe, Jr., Ed. Chicago, Year Book Publishers, 1950, vol. 2, p. 200.
18. Standardization of definitions and symbols in respiratory physiology. Fed. Proc. 1950, **9**, 602.
19. Kaltreider, N. L., Fray, W. W., and Hyde, H. V. Z. The effect of age on the total pulmonary capacity and its subdivisions. Amer. Rev. Tuberc. 1938, **37**, 662.
20. Needham, C. D., Rogan, M. C., and McDonald, I. Normal standards for lung volumes, intrapulmonary gas-mixing, and maximum breathing capacity. Thorax 1954, **9**, 313.
21. Whitfield, A. G. W., Waterhouse, J. A. H., and Arnott, W. M. The total lung volume and its subdivisions. A study in physiological norms. I. Basic data. Brit. J. soc. Med. 1950, **4**, 1.
22. Whitfield, A. G. W., Waterhouse, J. A. H., and Arnott, W. M. The total lung volume and its subdivisions. A study in physiological norms. III. Correlation with other anthropometric data. Brit. J. soc. Med. 1950, **4**, 113.
23. Bateman, J. B. Studies of lung capacities and intrapulmonary mixing; normal lung capacities. J. appl. Physiol. 1950, **3**, 133.
24. Gray, J. S., Grodins, F. S., and Carter, E. T. Alveolar and total ventilation and the dead space problem. J. appl. Physiol. 1956, **9**, 307.
25. Rossier, P. H., and Bühlmann, A. The respiratory dead space. Physiol. Rev. 1955, **35**, 860.
26. Fowler, W. S. Lung function studies. III. Uneven pulmonary ventilation in normal subjects and in patients with pulmonary disease. J. appl. Physiol. 1949, **2**, 283.
27. Bedell, G. N., Marshall, R., DuBois, A. B., and Comroe, J. H., Jr. Plethysmographic determination of the volume of gas trapped in the lungs. J. clin. Invest. 1956, **35**, 664.

TOTAL NONDIALYZABLE SOLIDS (TNDS) IN HUMAN URINE. I. THE AMOUNT AND COMPOSITION OF TNDS FROM NORMAL SUBJECTS [1]

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A number of high molecular weight compounds have been separated as constituents of normal urine. Regardless of the investigators' intention, in practically all of these studies only one or a few components were isolated. Interpretation and correlation of these numerous data, obtained by various procedures, would be greatly facilitated by the quantitative determination of the amount and composition of the total biocolloid content of normal 24 hour urine specimens. Hamerman, Hatch, Reife, and Bartz (1) have reported the recovery of the total nondialyzable solids (TNDS) from three 24 hour urine specimens by alternate dialysis and vacuum distillation with final lyophilization. The following experiments were designed to expand these data and to study individual variations in TNDS, in order to establish the limits of such variation in normal human urine.

MATERIALS AND METHODS

Subjects for the study were laboratory workers who were in excellent health, had no history or clinical evidence of renal or urinary tract disease, and who were within the average weight and height range for their age group. Each followed his daily routine without special diet, variation in fluid intake, or alteration in physical activity.

Twenty-four hour urine specimens were collected into chemically clean containers, with 10 ml. of phenylmercuric nitrate 1 : 1,000 as an inhibitor of enzymatic and bacterial activity. As soon as the collection was completed, each specimen was measured and the total specimen (or a 1,200 ml. aliquot) divided into 150 ml. portions. Each portion was placed in a 35 cm. Visking cellulose tubing [2] of 2.8 cm. inflated diameter. The sacs were knotted to include a 35 ml. volume of air in each bag. This arrangement kept the sac upright, absorbed mechanical shock, and permitted agitation of the urine during dialysis. The sacs were completely submerged in a closed carboy containing 20 liters of distilled water and dialyzed by brisk stirring at approximately 3° C. The distilled water was changed at daily intervals until the specific conductance of the urine was reduced to less than 9×10^{-5} mhos when measured at 0° C. in a Shedlovsky type closed cell with a constant of 14.3.

The sacs were emptied, with care being taken to flush out any solid which had separated from solution and adhered to the sides or bottom. The 24 hour specimens were then lyophilized in two steps, utilizing a Vertis freeze-drier of 7.8 liters capacity, with Cellosolve and solid carbon dioxide as the freezing mixture, under a pressure of approximately 0.1 mm. Hg. During the first step, 250 ml. portions of dialyzed urine in liter flasks were concentrated until the volume was approximately 25 ml. In the second step, the concentrates and rinsings were combined into a single flask and the lyophilization completed. Recovery of the solids could then be made with a minimum of mechanical loss.

The TNDS, a fluffy tan powder, were finally dried over anhydrous calcium sulfate in a vacuum desiccator to a constant weight. This required three to five days and the nonhygroscopic material thus obtained was utilized in the following studies.

Inorganic constituents. "Bound" water was determined by measurement of weight loss from the air-equilibrated TNDS after heating samples at 105 to 110° C. for two hours. The inorganic ash was calculated after ignition of 100 mg. samples at 500° C. for two to three hours. The calcium (2) and phosphorus (3) contents of the ash were determined by standard methods.

Protides. The nitrogen content of the TNDS was determined by a micro-Kjeldahl method utilizing Nessler's reagent prepared by the method of Koch and McMeekin (4). Samples of the TNDS from each subject were hydrolyzed and the amino acids present identified by two dimensional chromatography in three pairs of solvent systems, all as previously described (5).

Glucides. Total protein-bound hexose was estimated by both the orcinol (6) and anthrone (7) methods, utilizing standards of equal weights of galactose and mannose. Hexosamine was determined by the Schloss modification (8) of the Elson-Morgan reaction. Analyses for "sialic acid" were made by the diphenylamine technique (9), using as a standard either crystalline sialic acid [3]

[1] Supported by Mary Reynolds Babcock Foundation and Public Health Service Grants A-259, H-2820 and H-1998, National Institutes of Health.

[2] A. H. Thomas Co., dialyzer cellophane No. 4465-A2, 4.8 mμ pore size.

[3] Provided by Professor Richard J. Winzler, Department of Biochemistry, University of Illinois College of Medicine, Chicago, Illinois.

derived from human meconium or the sialic acid equivalent of the serum glycoprotein, orosomucoid.[3] All spectrophotometric measurements were made with the Beckman DU spectrophotometer.

Hydrolysis of the TNDS and unidimensional chromatography of the sugars were both performed by the technique of Glegg and Eidinger (10). A 48 hour hydrolysis period was used and double or triple ascensions of 40 cm. were made with each of three solvent systems, as previously described (5).

Lipids. Lipids were extracted from the TNDS with alcohol and alcohol-ether, purified with chloroform, and weighed according to Artom and Fishman (11). Total phospholipids were calculated from the phosphorus content (3) of the chloroform extract (lipid P × 25). Fatty acids were determined acidimetrically (12), either after direct saponification of the TNDS (subjects 2 and 4), or after fractionation of the lipid extract by repeated precipitations with acetone plus $MgCl_2$ and subsequent saponification of the precipitate and extract (pooled subjects 1, 6, 7 and 1, 1, 8). After titration of the fatty acids (total or acetone-soluble), the unsaponifiable matter was extracted and weighed. Total cholesterol was determined on the unsaponifiable matter either colorimetrically (13) or gravimetrically as the digitonin precipitate (14).

TABLE I

Inorganic and nitrogenous constituents of total nondialyzable solids of normal urine

Subject no.	Sex	Electrical conductivity* *mhos* $\times 10^{-6}$	TNDS *mg./24 hrs.*	% of TNDS			% of ash	
				"Bound" H_2O†	N	Ash	Ca	P
1	M	<5.7	413		8.1	6.3		
		40.0	453			6.6		
		91.0	497		6.5	6.3		
		21.0	191		7.1	8.0		
		<5.7	523		9.9	4.8	18.4	7.4
		<5.7	519		9.7	1.1	44.2	31.6
		<5.7	365		8.0	5.9	24.5	10.0
		12.0	304	13.0	8.7	3.9	22.8	7.6
			252					
			276					
		26.0	397					
		47.7	370					
2	M	65.0	508		6.4	4.6		
		36.0	505		8.6	7.1		
		<5.7	398		7.1	6.0	16.7	3.3
		16.0	393	13.0	8.6	4.9	20.1	12.2
		<5.7	364	11.5	9.2	4.1	27.8	4.2
		<5.7	374		8.3	4.9	19.1	10.5
		47.8	429		10.8	8.0	15.4	11.6
		20.0	447		9.5	7.6	6.5	2.8
			427		10.1			
3	M	<5.7	392		7.2	8.8	11.7	
		35.0	516		7.4	7.3	7.5	
4	M	21.0	633		6.8	8.2	7.2	12.0
		27.3	426		6.8	15.7	5.3	1.3
5	M	9.0	419	14.7	10.1	5.0	15.1	3.8
		37.6	626		8.5			
6	F	25.0	427		9.0	10.6		
		26.0	484		8.4	6.3		
		11.0	423		8.8	6.6	16.5	13.6
7	F	10.0	323		8.6	6.1	18.0	7.1
		<5.7	398		9.1	7.8	7.0	10.7
		55.0	617	8.0	10.7	7.2	9.3	15.4
8	F	26.5	330		7.2	15.6	4.5	4.0
		14.3	315		7.2	10.2	5.5	2.9
9	F	<5.7	305		8.9	7.1	22.7	7.8
		<5.7	292	13.1	9.0			

* Closed 1.0 ml. cell with constant of 14.3.
† Loss of water from air equilibrated solids at 105 to 110° C.

TABLE II

Glucide content of total nondialyzable solids of normal urine

Subject no.	TNDS mg./24 hrs.	Per cent of TNDS: Hexose: Anthrone	Hexose: Orcinol	"Sialic acid"	Hexosamine*
1	413	24.9	22.4	10.2	
	453	15.6	15.0	10.0	6.1
	497				6.7
	191	14.3	15.2	14.8	7.2
2	508	15.2	17.0	8.2	5.9
	505	17.6	17.5	9.3	
3	392	19.7	16.8	8.6	6.1
	516	13.9	14.3	8.5	5.9
6	427	16.0	14.8	7.6	
	484	15.7	16.5	9.9	5.5

* Expressed as glucosamine.

Tests for ketosteroids were made by a modification of the Zimmerman reaction (15). As little as 0.5 mg. of neutral 17-ketosteroids occurring in the TNDS of the 24 hour urine specimen would have been detectable by this procedure. An alcohol-chloroform-ether extract of a pooled TNDS sample was tested for 17, 21-dihydroxy-20-ketosteroids by the Porter-Silber test (16).

RESULTS

In Tables I, II, and III are presented the values for inorganic constituents, total N, glucides and lipids, determined on the TNDS of 37 24 hour urine specimens from nine subjects. These values represent the averages of duplicate or triplicate determinations and are expressed as dry weight percentage of lyophilized TNDS. In Table IV are recorded the average excretions of the various constituents, together with the standard deviations of these averages. Also in Table IV, a comparison has been made between the variability of the amounts for the whole group and that for two individual subjects.

Inorganic constituents

Calcium and phosphorus make up a variable percentage of the total ash of the TNDS (4.5 to 44.2 per cent and 1.3 to 31.6 per cent, respectively). However, the 24 hour rate of excretion of these two constituents is much more constant, both for the group of subjects and for subject No. 2 (Table IV). Tests for inorganic phosphorus were negative prior to ashing, although calcium could be measured equally well on the unashed material. There was no consistent calcium to total phosphorus ratio for the TNDS. Also, the lipid P : N ratio was inconsistent, but the lipid P : ash P ratio was about 1 : 25. It will be seen in Table IV that the average excretion of ash for subjects Nos. 1 and 2 was significantly lower than in the group as a whole. No obvious explanation is apparent for this finding. In all other respects studied, there was no significant difference between the values obtained from these two subjects and those for the group as a whole.

Protides

The nitrogen content of the TNDS averaged 8.4 per cent, corresponding to the excretion of 36.2 ± 11.5 mg. in 24 hours. When the nitrogen content of the TNDS is corrected for the nitrogen

TABLE III

Lipids of total nondialyzable solids of normal urine

Subject no.	TNDS mg./24 hrs.	Per cent of TNDS: Total lipids	Total phospholipids*	Fatty acids: Total	Fatty acids: Phospholipid	Unsaponifiable: Total	Unsaponifiable: Cholesterol
2	505			1.13		1.46	0.72†
2	398	2.24	0.22				
4	633	4.26	1.04				0.67†
4	426	2.89	0.16				
6	427	4.27	0.98				
1, 6, 7‡	Pooled	3.52	0.95	1.57§	0.74	1.49	0.98‖
1, 1, 8‡	Pooled	2.51	0.76	0.81§	0.42	1.36	

* Lipid P × 25.
† Colorimetric method (13).
‡ Pooled samples.
§ Phospholipid fatty acids, plus fatty acids. Soluble in acetone.
‖ Gravimetric method (14).

TABLE IV

Average excretion of inorganic and organic constituents of total nondialyzable solids of normal urine *

	TNDS	Ash	Calcium	Phosphorus	Nitrogen	Hexose (orcinol)	Sialic acid	Hexosamine	Total lipids
Number of subjects	7	7	8	7	7	4	4	4	6
Number of determinations	16	14	16	14	16	9	9	7	6
Mean values	433	36.7	3.5	2.4	36.2	72.6	40.7	26.5	15.6
Standard deviation	114.6	13.2	1.1	2.0	11.5	19.1	7.5	6.1	8.1
				Subject number 1					
Number of determinations	12	8			8				
Mean values	380	20.9†			35.0				
Standard deviation	108.4	9.0			12.8				
				Subject number 2					
Number of determinations	9	8	6	6	8				
Mean values	427	25.4‡	3.8	1.8	36.5				
Standard deviation	52.2	8.2	1.0	1.2	6.7				

* Expressed as mg. per 24 hours.

† The difference between this value and that for the group was tested by the "t" test. The probability is $<0.01 > 0.001$.

‡ The difference between this value and that for the group was tested by the "t" test. The probability is $<0.05 > 0.02$.

content of amino sugars (7.8 per cent) and sialic acid (3.79 per cent) and then multiplied by the factor 6.25, an estimate of 47 per cent protein content is obtained for the TNDS, equivalent to 204 mg. of protein per 24 hour sample.

The following amino acids were identified by chromatography, arranged in decreasing order of color intensity after development with ninhydrin: aspartic and glutamic acids, alanine, leucine, isoleucine, valine, phenylalanine, glycine, serine, threonine, lysine, arginine, proline, tryptophan, and tyrosine. The last two were very weak and not consistently seen in all chromatograms. Histidine, cystine, cysteine, methionine, taurine, tryptamine, ornithine, hydroxyproline, and hexosamine were not identified by chromatography. This is not conclusive evidence for their absence, since hydrolytic destruction or alteration of certain compounds may have occurred, especially in the presence of carbohydrates.

Three unidentified ninhydrin-positive spots were found repeatedly. The approximate R_F values in each direction of two of the unidentified spots were, respectively, 0.13 and 0.06, and 0.15 and 0.72, in the lutidine-phenol system (5), 0.50 and 0.07 for the single unknown spot seen in the butanol: acetic acid-butanol: ethanol system (5), and 0.24 and 0.03, 0.20 and 0.12, and 0.01 and 0.08 for three unidentified spots seen in the propanol: ethylacetate-isobutyric acid system (5). The hydrolysis was considered to be strong enough to eliminate the possibility of these being peptides.

Glucides

Values for total protein-bound hexose obtained by the anthrone and orcinol techniques were in excellent agreement (Table II). When examined by chromatography, the resin-hydrolyzed (10) material consistently gave five spots which migrated on all chromatograms identically with galactose, mannose, glucose, rhamnose, and fucose, in respective order of decreasing color intensity. A "specific" test (17) indicated that less than 0.05 per cent ketohexose was present in TNDS, and no maximum absorption at 560 mμ was noted when the method of Dische and Borenfreund (18) was applied to the TNDS for the detection of ketoses.

The average value of 9.7 per cent for sialic acid content of the TNDS is considered to be reliable. When the absorption curves for the products of the reactions with TNDS, orosomucoid, and crystalline sialic acid were plotted over the range of 300 to 900 mμ, identical maxima at 525 and 712 mμ and minima at 450 and 577 mμ were obtained for all these substances.

Lipids

The presence of small amounts of lipids in normal human urine was first noted by Mörner (19)

and has been reported subsequently by many investigators [see literature in Bloor (20)]. The content of total lipids, total phospholipids, fatty acids, and unsaponifiable material in the TNDS is rather variable (Table III). The values for total lipids and cholesterol, calculated as mg. per 24 hours, found in the various specimens are within the range of values for undialyzed urine given by previous authors (20). The chloroform soluble phosphorus of the TNDS exhibited much greater individual variations than did the total lipids. It appears that the chloroform soluble phosphorus represents chiefly the glycerolphospholipids, which contain approximately 4 per cent phosphorus and 67 per cent fatty acids. Indeed, if these percentages are used as factors, similar values are obtained for the total phospholipids calculated either from the lipid phosphorus or from the fatty acid content of the acetone precipitate. Thus, in the pooled samples from subjects 1, 6, and 7, the total phospholipids are calculated as 9.5 mg. per Gm. on the basis of phosphorus content and as 11.2 mg. per Gm. on the basis of fatty acid content. Likewise, in the pooled samples from subjects 1, 1, and 8 the corresponding values are 7.6 and 6.2 mg. per Gm., respectively.

Steroid and other analyses

Tests for neutral 17-ketosteroids and 17,21-dihydroxy-20-ketosteroids were negative. The quantitative Aschheim-Zondek test revealed the presence of gonadotrophic hormone when TNDS were administered intravenously to female rabbits. The diuretic factor of Little (21) is present in this material. Type specific sera have demonstrated the presence of blood group specific substances A, B, and O in the TNDS (1, 22).

DISCUSSION

On the basis of these data, it appears that the average composition of TNDS of normal urine is approximately: 47 per cent protein, 16.6 per cent protein-bound hexose, 9.7 per cent sialic acid, 6.2 per cent hexosamine, 3.3 per cent lipid, 12.2 per cent water, and 8.5 per cent ash. These average figures account for 103.5 per cent of the TNDS. Both the relative composition and amounts of the TNDS excreted are rather constant in the normal individual, when one considers the vast number of metabolic processes that may be represented by this material. The present data are too few to permit unequivocal conclusions, but no marked differences in TNDS have been observed between male and female or between white and Negro subjects. The pathways by which these materials reach the urine are probably a combination of glomerular filtration, urinary epithelial secretion, and possibly renal tubular secretion. The contribution of transitional epithelial cells and blood cells to the TNDS remains unknown. Microscopic examination of the residue after dialysis and subsequent ultrafiltration revealed no evidence of any formed cell, cast or other morphological element. Dialysis thus resulted in the lysis of the cellular constituents so that their total contribution to the TNDS could be only the nondialyzable portion of the cells. This quantity cannot be determined at the present time, since there is no obvious way of separating these materials from the TNDS. Centrifugation at the speed required for an Addis count will also remove a variable quantity of mucoprotein from normal urine (23). Mucoprotein so removed may vary from 5 to 50 mg. per 24 hour specimen, depending upon the specific gravity, salt content, and pH of the urine. Presumably, the error introduced by such a procedure in the final weight of the TNDS would be much greater than any error which could be expected to result from the presence of nondialyzable cellular residue in normal urine.

The dialysis was intended to achieve a maximal separation of the diffusible materials with minimal decomposition of the TNDS. The duration of the dialysis adopted for these studies appeared to be adequate, since there was no relationship between the observed conductance and the quantity of TNDS (Table I). Also, the fact that the amount of ash in the 24 hour TNDS is rather constant (Table IV) is an indication of the reproducibility of the conductance-controlled dialysis and of its efficiency, since the theoretical ratio of dialyzable to nondialyzable material in normal urine is approximately 150:1. It seems necessary to adopt some arbitrary criterion such as a definite value of electrical conductance as an end point for the dialysis. This is an asymptotic process which causes a continual disturbance of the equilibria

between bound and unbound molecules in the urine. Thus, whereas calcium may be bound in normal urine to some poorly diffusible molecules (24, 25), in the present study the excretion of nondialyzable calcium is relatively constant (Table IV). This was possible because a definite, although arbitrary, criterion was adopted to terminate dialysis. If other criteria had been selected, other values for the excretion of nondialyzable calcium might have been obtained.

Similarly, hexuronic acid (26, 27) and hexosamine (28, 29) exist in urine in both dialyzable and nondialyzable [4] forms; accordingly, the detection and estimation of these compounds in the TNDS will depend to some extent upon the conditions of dialysis. It is impossible to determine by conventional techniques the exact amount of hexuronic acid in the TNDS since it is relatively small, particularly susceptible to hydrolytic destruction, and is also progressively removed by dialysis. Hydrolysis of the TNDS with resin (10) or by the procedure of DeFrates and Boyd (30) resulted in a clear, pale yellow hydrolysate which gave a clearly positive naphthoresorcinol test for hexuronic acid. However, we were unable to detect hexuronic acid after the more severe hydrolysis described by Hamerman, Hatch, Reife, and Bartz (1) nor did the characteristic absorption peak appear with this material after reaction with thioglycolic acid (31), although both chondroitin sulfate and material isolated from urine by the procedure of Di Ferrante and Rich (27) gave positive tests for hexuronic acid under the same conditions. Therefore, no attempt was made to estimate the quantity of hexuronic acid in TNDS, since the result could have been only a reflection of the reproducibility of the hydrolytic conditions. On the other hand, the separation of hexosamine as an integral part of the molecule from that which is free or loosely bound to the TNDS appears to have been clearly accomplished. A positive test for hexosamine was observed only on the hydrolyzed material. Comparison of the results for hexosamine with those of other investigators (1, 28, 29) suggests that the hexosamine content of TNDS of normal urine falls within reasonably reproducible limits.

[4] The term "bound" is inappropriate since molecules having molecular weights up to 20,000 can pass Visking cellophane membranes of the kind used here, and some of these compounds probably contain hexuronic acid and/or hexosamine.

SUMMARY

The total nondialyzable solids (TNDS) of normal human urine specimens have been recovered by lyophilization and determined to be 433 mg. per 24 hours, with a standard deviation of 114.6. The TNDS were analyzed for inorganic ash, bound water, calcium, phosphorus, nitrogen, hexose, sialic acid, hexosamine, total lipids, total phospholipids, fatty acids, and unsaponifiable matter. The approximate composition of the TNDS is: 47 per cent protein, 16.6 per cent protein-bound hexose, 9.7 per cent sialic acid, 6.2 per cent hexosamine, 3.3 per cent lipids, 12.2 per cent "bound" water, and 8.5 per cent ash. With respect to these constituents, there was no significant variation among normal adult subjects. The amino acids and sugars were studied by paper chromatography. The excretion for some of the nondialyzable constituents, expressed as mg. per 24 hours, follows: inorganic ash, 36.7 ± 13.2; calcium, 3.5 ± 1.1; phosphorus, 2.4 ± 2.0; nitrogen, 36.2 ± 11.5; hexose, 72.6 ± 19.1; sialic acid, 40.7 ± 7.5; hexosamine, 26.5 ± 6.1; and total lipids, 15.6 ± 8.1. Each of these values was obtained from four to eight subjects who submitted a total of 7 to 16 24 hour urine specimens.

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REFERENCES

1. Hamerman, D., Hatch, F. T., Reife, A., and Bartz, K. W. Nondialyzable material in normal human urine. J. Lab. clin. Med. 1955, **46**, 848.
2. Clark, E. P., and Collip, J. B. A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. biol. Chem. 1925, **63**, 461.
3. Fiske, C. H., and Subbarow, Y. The colorimetric determination of phosphorus. J. biol. Chem. 1925, **66**, 375.
4. Hawk, P. B., Oser, B. L., and Summerson, W. H. Practical Physiological Chemistry, 12th ed. Philadelphia, Blakiston, 1947, p. 547.
5. King, J. S., Jr., and Boyce, W. H. Amino acid and carbohydrate composition of the mucoprotein ma-

trix in various calculi. Proc. Soc. exp. Biol. (N. Y.) 1957, **95**, 183.
6. Weimer, H. E., and Moshin, J. R. Serum glycoprotein concentrations in experimental tuberculosis of guinea pigs. Amer. Rev. Tuberc. 1952, **68**, 594.
7. Scott, T. A., Jr., and Melvin, E. H. Determination of dextran with anthrone. Analyt. Chem. 1953, **25**, 1656.
8. Schloss, B. Colorimetric determination of glucosamine. Analyt. Chem. 1951, **23**, 1321.
9. Winzler, R. Determination of serum glycoproteins *in* Methods of Biochemical Analysis, D. Glick, Ed. New York, Interscience, 1955, vol. 2, p. 298.
10. Glegg, R. E., and Eidinger, D. Hydrolysis of polysaccharides by a cation exchange resin and identification of monosaccharide components by paper chromatography. Analyt. Chem. 1954, **26**, 1365.
11. Artom, C., and Fishman, W. H. The relation of the diet to the composition of tissue phospholipids. I. The normal composition of liver and muscle lipids of the rat, with a note on the analytical procedures. J. biol. Chem. 1943, **148**, 405.
12. Artom, C. Remarques sur le dosage acidimétrique des acides gras dans les tissus parenchymateux. Bull. Soc. Chim. biol. (Paris) 1932, **14**, 1386.
13. Bloor, W. R. The determination of cholesterol in blood. J. biol. Chem. 1916, **24**, 227.
14. Caminade, R. Sur quelques modifications apportées au dosage de la cholestérine par la méthode de Windaus. Bull. Soc. Chim. biol. (Paris) 1922, **4**, 601.
15. Callow, N. H., Callow, R. K., and Emmens, C. W. Colorimetric determination of substances containing the grouping $-CH_2 \cdot CO-$ in urine extracts as an indication of androgen content. Biochem. J. 1938, **32**, 1312.
16. Porter, C. C., and Silber, R. H. A quantitative color reaction for cortisone and related 17,21-dihydroxy-20-ketosteroids. J. biol. Chem. 1950, **185**, 201.
17. Tauber, H. A specific qualitative color test for ketohexoses. J. biol. Chem. 1950, **182**, 605.
18. Dische, Z., and Borenfreund, E. A new spectrophotometric method for the detection and determination of keto sugars and trioses. J. biol. Chem. 1951, **192**, 583.
19. Mörner, K. A. H. Untersuchungen über die Proteinstoffe und die eiweissfällenden Substanzen des normalen Menschenharns. Skand. Arch. Physiol. 1895, **6**, 332.
20. Bloor, W. R. Biochemistry of the Fatty Acids. New York, Reinhold, 1943, p. 372.
21. Little, J. M. Fractionation of the diuretic factor present in pregnant mare urine. Amer. J. Physiol. 1955, **180**, 173.
22. Boyce, W. H. Unpublished data.
23. Boyce, W. H., and Swanson, M. Biocolloids of urine in health and in calculous disease. II. Electrophoretic and biochemical studies of a mucoprotein insoluble in molar sodium chloride. J. clin. Invest. 1955, **34**, 1581.
24. Vermeulen, C. W., Miller, G. H., and Chapman, W. H. Experimental urolithiasis: X. On the state of calcium in the urine. J. Urol. (Baltimore) 1956, **75**, 592.
25. Neuman, W. F., and Neuman, M. W. Emerging concepts of the structure and metabolic functions of bone. Amer. J. Med. 1957, **22**, 123.
26. Kerby, G. P. The occurrence of acid mucopolysaccharides in human leucocytes and urine. J. clin. Invest. 1955, **34**, 1738.
27. Di Ferrante, N., and Rich, C. The determination of acid aminopolysaccharide in urine. J. Lab. clin. Med. 1956, **48**, 491.
28. Boas, N. F. Low molecular weight hexosamine-containing compounds in urine. Proc. Soc. exp. Biol. (N. Y.) 1956, **92**, 122.
29. King, J. S., Jr., and Warnock, N. H. Some clinical conditions affecting urinary excretion of non-dialyzable hexosamine. Proc. Soc. exp. Biol. (N. Y.) 1956, **92**, 369.
30. DeFrates, J. S., and Boyd, M. J. Microdetermination of glucuronic acid in blood. Fed. Proc. 1953, **12**, 194.
31. Dische, Z. A specific color reaction for glucuronic acid. J. biol. Chem. 1947, **171**, 725.

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